MODULATION OF SAPONIN PRODUCTION IN MEDICINAL *MAESA* SPP.

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Cover art: *Saponin rain* by Hanifah Siregar | www.flickr.com/photos/nipasir

Modulation of saponin production in medicinal *Maesa* spp.

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Thesis submitted in fulfillment of the requirements
for the degree of Doctor (PhD) in Applied Biological Sciences

Academic year: 2012-2013
Promotor: Prof. dr. Danny Geelen

Faizal, A (2013) Modulation of saponin production in medicinal *Maesa* spp. PhD Thesis, Ghent University, Ghent, Belgium.

ISBN-number: 978-90-5989- 599-7

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Contents

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
αAS	α-amyrin synthase
ABA	abscisic acid
AFLP	amplified length fragment polymorphism
ANOVA	analysis of variance
AUX1	auxin1
BA	benzyladenine
bAS	beta-amyrin synthase
Bf	*Bupleurum falcatum*
BLAST	basic local alignment search tool
bp	base pair
cDNA	complementary DNA
Combiplan	combinatorial biosynthesis in plants
CS	cycloartenol synthase
CSI	chromosaponin 1
CTR	control
CV	coefficient of variation
CypP450	cytochrome P450
Da	Dalton
DAPI	4',6' diamino-2-phenylindole
DMAPP	dimethylallyl diphosphate
DNA	deoxyribonucleic acid
dpi	days post infiltration
dsRNA	double-stranded RNA
EcR	ecdysteroid receptor
eGFP	enhanced GFP
ENOD	*early nodulin*
ER	endoplasmic reticulum

ESI	electrospray ionization
FL-ORF	full length-open reading frame
FPP	farnesyl diphosphate
FPS	farnesyl diphosphate synthase
FT-ICRMS	Fourier transform-ion cyclotron resonance mass spectrometry
GA3	gibberellic acid
GFP	green fluorescent protein
Gg	*Glycyrrhiza glabra*
GPP	geranyl diphosphate
GPS	geranyl diphosphate synthase
GT	glycosyltransferase
GUS	β-glucuronidase
HPLC	high performance liquid chromatography
HR	hairy root
IBA	indole-3-butyric acid
IPP	isopentenyl diphosphate
IT	ion trap
JA	jasmonic acid
Kin	kinetin
LC	liquid chromatography
LuS	lupeol synthase
MB	maesabalide
MeJA	methyl jasmonate
MEP	2-C-methyl-D-erythritol 4-phosphate
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog (macro and micro elements)
	mass spectrometry
	maesasaponin
Ml	*Maesa lanceolata*
Mt	*Medicago truncatula*

MVA	mevalonic acid
MW	molecular weight
NAA	α-naphthalene acetic acid
NADH	nicotinamide adenine dinucleotide hydrogen
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NPT	neomycin phosphotransferase
OD	optical density
OSC	oxidosqualene cyclase
PCR	polymerase chain reaction
Pg	*Panax ginseng*
PGR	plant growth regulator
PMT	photomultiplier
PTGS	post-transcriptional gene silencing
PVP-10	polyvinylpyrrolidone 10000
RDR6	RNA-dependent RNA polymerase6
R_f	retention factor
Ri	root inducing
RISC	RNA-inducing silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rotations per minute
R_T	retention time
RT-PCR	reversed transcriptase PCR
SA	salicylic acid
Sad	saponin-deficient
SD	standar deviation
SEM	standard error of the mean
SH	Schenk and Hildebrandt
SIM	shoot induction medium
siRNA	small interfering RNA

TBSV	*Tomato bushy stunt virus*
T-DNA	transfer DNA
TDZ	thidiazuron
TGS	transcriptional gene silencing
Ti	tumor inducing
TLC	thin layer chromatography
Trolox	6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid
UPLC	ultra performance liquid chromatography
UV	ultra violet
vir	virulence
v/v	volume/volume
w/v	weight/volume

Scope

A myriad of secondary metabolites synthesized by plants is an invaluable asset for the discovery of novel bioactive molecules that may be developed as pharmaceuticals. Saponins are a group of chemicals that occur in a wide range of plant species and exhibit many different biological activities that are hitherto underexplored. To fully exploit this potential, we need to improve the purification and synthesis and understand how production is regulated in plants. Accordingly, in **Chapter 1** a review about saponins biosynthesis, distribution and localization in plants as well as their roles is presented.

The main goal of this thesis was to modulate saponin production from four *Maesa* species, *Maesa argentea*, *M. balansae*, *M. lanceolata*, and *M. perlarius.* These species were selected based on previous ethnopharmacology investigations and potential medicinal properties. These species have been widely used by traditional healers to treat diseases in Asia and Africa. Further evaluations lead to the identification of saponins with an important role in the future treatment of leishmania and cancer. To support this investigation, in **Chapter 2** we established an effective means for the in vitro cultivation and propagation not only to ensure a continuous supply of plant materials, but also to build a platform for further in vitro research, such as genetic transformation and investigation of the gene expression in saponin biosynthesis. In addition to in vitro materials, we also evaluated saponin production from greenhouse plants in **Chapter 3**. Another aim of this project was to contribute to a combinatorial biosynthesis platform in plants with the triterpene saponins as the target**.** This could be achieved by heterologous expression of the candidate saponin biosynthesis genes in *M. lanceolata* hairy roots as described in **Chapter 4**. Besides a hairy root system, in **Chapter 5** we also developed an agroinfiltration technique, using *Agrobacterium tumefaciens*-mediated transformation as an alternative approach to regenerate stably transgenic plants. By applying this technique in **Chapter 6**, we were able to change the β-amyrin synthase expression, one of the key precursors of the triterpene saponin pathway and assess the impact on saponin production of *M. lanceolata*. Finally, we also attempted to modulate saponin production by in vitro polyploidization as described in **Chapter 7**. For this, a callus regeneration protocol was developed and the genome doubling effect on saponin production from *M. perlarius* was also evaluated.

1 | Saponins and their role in plants

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Introduction: Saponins and their role in plants

Publication status:

Ahmad Faizal and Danny Geelen. **Saponins and their role in biological processes in plants**.

Manuscript was submitted for publication

Abstract

Saponins are steroid and triterpenoid glycosides that display diverse biological activities. The wide-spread occurrence in plants as well the potential for pharmaceutical application has lead to saponin extraction and identification from numerous species. Although these efforts are important to extend our knowledge of naturally occurring saponin structures, recent attention has been given to the biosynthesis and distribution in plants. Here, we present recent advances on saponin production and distribution and highlight studies showing the effects on growth and development.

1. Introduction

Saponins are structurally complex amphiphatic glycosides of steroids and triterpenoids that are widely produced by plants (Sparg et al., 2004; Vincken et al., 2007) and also by certain marine organisms, such as starfish and sea cucumbers (Tang et al., 2009; Van Dyck et al., 2010). Their name is derived from Latin s*apo* meaning soap, because they have surfactant properties forming stable soap-like foam upon shaking in aqueous solution. Chemically, the term saponin defines a group of high molecular weight glycosides that consist of a glycan moiety linked to an aglycon which is also called genin or sapogenin (Hostettmann and Marston, 1995). The chemical structures of sapogenin defines the classification of saponins as triterpenoid saponins (30 carbon atoms) (Fig. 1a). These triterpenoid saponins occur mainly in the class of Magnoliopsida, while steroidal saponins (27 carbon atoms with a 6-ring spirostane or a 5-ring furostane skeleton) (Fig. 1b)are almost exclusively present in the class of Liliopsida (Sparg et al., 2004) (Fig. 1). According to the carbon skeleton of the aglycon, saponins are sometimes further classified into 12 main classes, namely the: dammaranes, tirucallanes, lupanes, hopanes, oleananes, 23-nor oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroids (Vincken et al., 2007). Moreover, saponins are often present as a complex mixtures and their composition may vary depending on the genetic background, the tissue type, the age and the physiological state of the plant, and environmental factors (Szakiel et al., 2011a).

Saponins as plant secondary metabolites are different from the components of primary metabolism because they are generally non essential for basic metabolic processes in plant. Instead saponins are part of a diversity of secondary metabolites that have been clearly demonstrated to play a role in the adaptation of plants to their environment. The production of these secondary molecules may be part of the response to external factors including various biotic and abiotic stimuli. In addition, they contribute to the innate immunity as phytoprotectants including phytoanticipins which are constitutively produced and inducible phytoalexins (Dixon, 2001).

Saponins have also been studied for their wide range of properties, including beneficial and detrimental effects on human health, pesticidal, insecticidal, molluscicidal and fungicidal activity, bitterness and sweetness and other industrial applications such as foaming and surface active agents. The pharmacological activities have been reviewed

extensively (Lacaille-Dubois and Wagner, 1996; Francis et al., 2002; Sparg et al., 2004; Park et al., 2005; Augustin et al., 2011). In this review, we present recent advances on saponin production and distribution in plants and their possible roles in biological processes including developmental regulation.

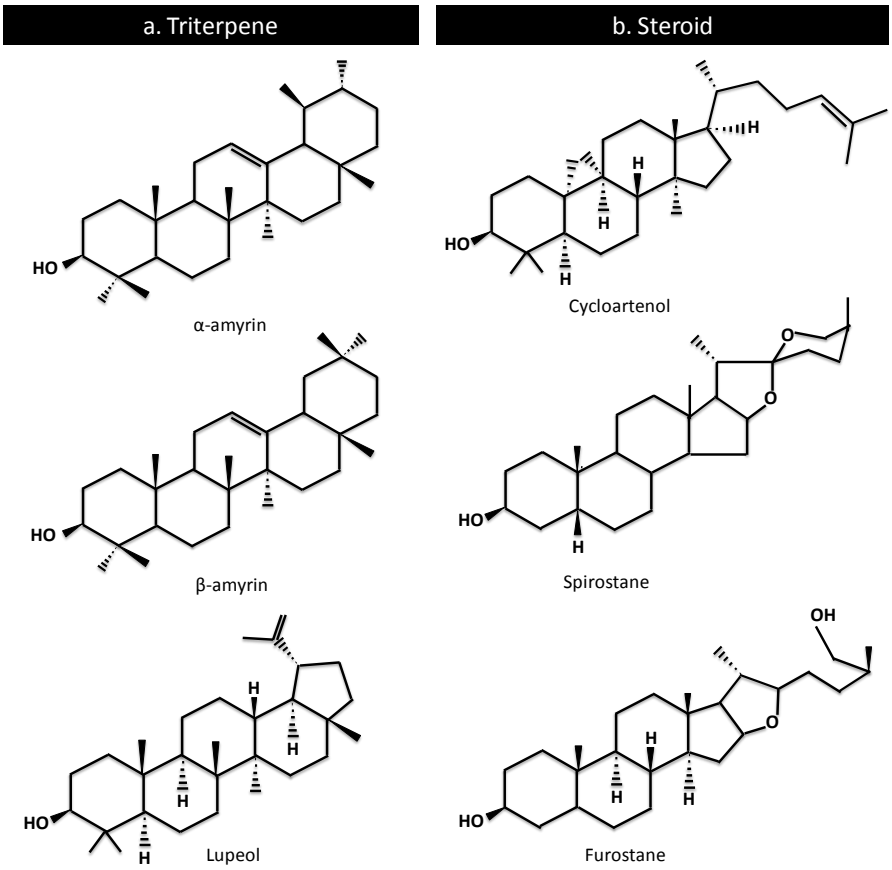

Fig. 1 The common classes of triterpene (a) and steroid (b) sapogenins

2. Biosynthesis of saponins

Saponin backbones are synthesized via the isoprenoid pathway involving a number of unidentified enzymatic steps (Fig. 2). What is known is that squalene (30-carbon intermediate), which is a precursor for both triterpenoid and steroidal saponins is produced from the mevalonate and non-mevalonate [2-*C*-methyl-D-erythritol 4-phosphate (MEP)] pathways through a series of enzymatic reactions involving geranyl diphosphate synthase (GPS), farnesyl diphosphate synthase (FPS) and squalene synthase (Misawa, 2011). Subsequently, squalene is transformed into 2,3-oxidosqualene by squalene epoxidase. The

cyclization of 2,3-oxidosqualene by oxidosqualene cyclases (OSCs) leads to production of tetracyclic sterols through the activity of cycloartenol synthase (CS). Furthermore, steroidal saponins are thought to derive from intermediates in phytosterol pathway downstream of cycloartenol formation. However, the steps at which steroidal saponins and phytosterol biosynthesis diverge have not been elucidated, although cholesterol has been suggested as a candidate precursor of steroidal saponins (Phillips et al., 2006; Vincken et al., 2007).

Triterpene saponin biosynthesis branches off the phytosterol pathway by alternative cyclization of 2,3-oxidosqualene through the activity of enzymes such as β-amyrin synthase (βAS), α-amyrin synthase (αAS), and lupeol synthase (LuS) (Haralampidis et al., 2002; Vincken et al., 2007). Following cyclization, further diversity is obtained by modification of the products through oxidation, hydroxylation, glycosylation, and other substitutions mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases (GTs), and other enzymes (Fig. 2). However, not much is known about the enzymes required for these chemical reactions.

One common feature shared by all saponins is the presence of a sugar chain attached to the aglycon. Glycosylation is important as the sugar chain is crucial for biological activities of saponins. The oligosaccharide chains are likely to be synthesized by sequential addition of single sugar residues to the aglycon but there is little experimental data about the mechanism of saponin glycosylation. The genetic machinery required for the elaboration of these saponins is as yet largely uncharacterized, despite the considerable commercial interest in this important group of natural products. This is likely to be due in part to the complexity of the molecules and the lack of pathway intermediates for biochemical studies.

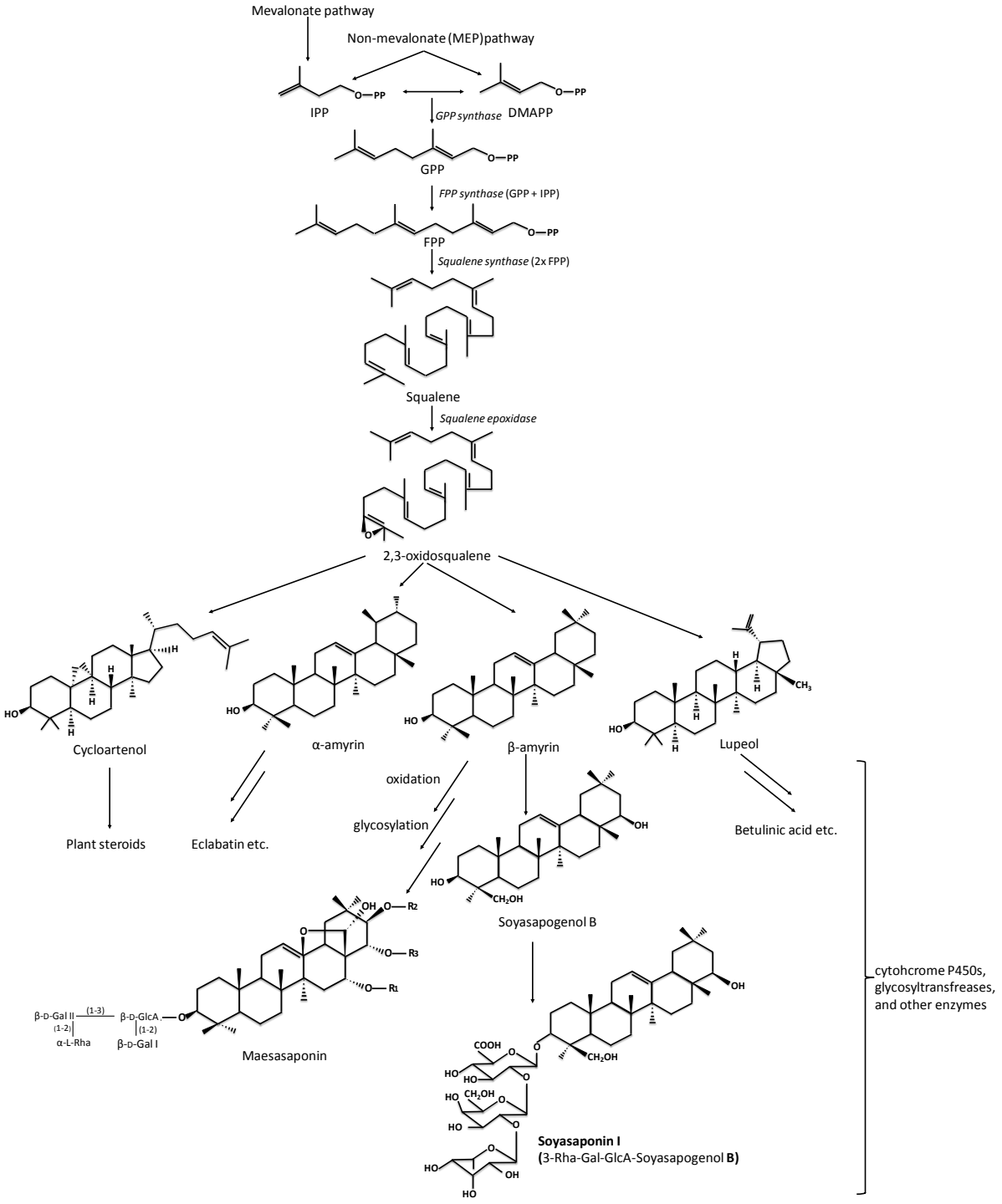

Fig. 2 Biosynthesis of saponins and their structural diversification (IPP – isopentenyl diphosphate, DMAPP – dimethylallyl diphosphate, GPP – geranyl diphosphate, FPP – farnesyl diphosphate, Gal – galactose, GlcA – glucuronic acid, Rha – rhamnose).

3. The occurrence of saponins in plants

Saponin producing plants are found in various geographical regions and climatic zones around the world. These include annual and biennial herbs, grasses, perennial evergreen, shrubs, trees, and wild and cultivated species. Table 1 provides a list of saponin research in the last 4 years (2008-2012) across many different plant families. Although the list is not complete, it does give a good overview of the plant species and families which have been the focus of saponin research in recent years. Of the approximately 100 species listed, about half of these have been investigated for biological activities. Furthermore, a number of species listed were selected based on previous ethnobotanical studies on related species from the same genus or family, for example genus *Chlorophytum* from the family Liliaceae. Another example is genus *Maesa* from family Primulaceae. They were investigated because their saponins were reported to have anti-cancer and anti-leishmania activities (Apers et al., 2002; Germonprez et al., 2005).

Table 1 A list of plant species from which saponins have been isolated in 4 recent years (2008 – 2012). Earlier report can be found in Sparg et al. (2004) and Dinda et al. (2010)

Family	Species	Saponin type	Reference
Class of Magnoliopsida			
Amaranthaceae	*Achyranthes fauriei*	Triterpenoid	Ando et al. (2008)
	Alternanthera philoxeroides	Triterpenoid	Fang et al. (2009)
	Celosia cristata	Triterpenoid	Wang et al. (2010)
Araliaceae	*Aralia elata*	Triterpenoid	Lee et al. (2009)
	Cussonia arborea	Triterpenoid	Kougan et al. (2009)
	Hydrocotyle bonariensis	Triterpenoid	Tabopda et al. (2012)
	Hydrocotyle sibthorpioides	Triterpenoid	Huang et al. (2008)
	Meryta denhamii	Triterpenoid	Cioffi et al. (2008)
Apiaceae	*Eryngium yuccifolium*	Triterpenoid	Zhang et al. (2008)
	Physospermum verticillatum	Triterpenoid	Tundis et al. (2009)
Aquifoliaceae	*Ilex kudingcha*	Triterpenoid	Zuo et al. (2012)
	Ilex pubescens	Triterpenoid	Wang et al. (2008)
Asteraceae	*Aster sedifolius*	Triterpenoid	Cammareri et al. (2008)
	Lactuca scariola	Triterpenoid	Yadava and Jharbade (2008)
	Silphium radula	Triterpenoid	Calabria et al. (2008)
Balsaminaceae	*Impatiens siculifer*	Triterpenoid	Li et al. (2009)
Bignoniaceae	*Incarvillea delavayi*	Triterpenoid	Ge et al. (2009)
Brassicaceae	*Barbarea vulgaris*	Triterpenoid	Nielsen et al. (2010)
Caesalpiniaceae	*Cassia angustifolia*	Triterpenoid	Khan and Srivastava (2009)
Campanulaceae	*Codonopsis lanceolata*	Triterpenoid	Shirota et al. (2008)

Caprifoliaceae	*Lonicera macranthoides*	Triterpenoid	Chen et al. (2009)
Caryophyllaceae	*Dianthus superbus*	Triterpenoid	Chen et al. (2010)
	Dianthus versicolor	Triterpenoid	Ma et al. (2009)
	Gypsophila altissima	Triterpenoid	Chen et al. (2010)
	Gypsophila pacifica	Triterpenoid	Nie et al. (2010)
	Gypsophila pilulifera	Triterpenoid	Arslan et al. (2012)
	Silene viscidula	Triterpenoid	Xu et al. (2011)
	Psammosilene tunicoides	Triterpenoid	Deng et al. (2009)
Chenopodiaceae	*Salicornia herbacea*	Triterpenoid	Kim et al. (2012)
Chrysobalanaceae	*Licania arianeae*	Triterpenoid	de Carvalho et al. (2008)
Combretaceae	*Terminalia ivorensis*	Triterpenoid	Ponou et al. (2010)
	Terminalia tropophylla	Triterpenoid	Cao et al. (2010)
Convovulaceae	*Ipomoea batatas*	Triterpenoid	Dini et al. (2009)
Euphorbiaceae	*Glochidion eriocarpum*	Triterpenoid	Kiem et al. (2009)
Fabaceae	*Abrus precatorius*	Triterpenoid	Xiao et al. (2012)
	Albizia inundata	Triterpenoid	Zhang et al. (2011)
	Caragana microphylla	Triterpenoid	Jin et al. (2011)
	Gleditisia sinensis	Triterpenoid	Gao et al. (2008)
	Medicago Arabica	Triterpenoid	Tava et al. (2009)
	Pueraria lobata	Triterpenoid	Niiho et al. (2010)
	Pueraria thomsonii	Triterpenoid	Niiho et al. (2010)
Iridaceae	*Crocus sativus*	Triterpenoid	Rubio-Moraga et al. (2011)
Lecythidaceae	*Stauntonia chinensis*	Triterpenoid	Gao et al. (2008)
Loganiaceae	*Antonia ovata*	Triterpenoid	Magid et al. (2010)
Mimosaceae	*Cylicodiscus gabunensis*	Triterpenoid	Tene et al. (2011)
	Tetrapleura tetraptera	Triterpenoid	Note et al. (2009)
Myrsinaceae	*Ardisia gigantifolia*	Triterpenoid	Wen et al. (2008)
	Ardisia kivuensis	Triterpenoid	Ndontsa et al. (2012)
	Ardisia pusilla	Triterpenoid	Tian et al. (2009)
Phytolaccaceae	*Phytolacca americana*	Triterpenoid	Wang et al. (2008)
	Phytolacca bogotensis	Triterpenoid	Montoya et al. (2009)
Polygalaceae	*Polygala crotalarioides*	Triterpenoid	Hua et al. (2010)
	Polygala tenuifolia	Triterpenoid	Li et al. (2008)
	Securidaca longepedunculata	Triterpenoid	Mitaine-Offer et al. (2010)
Primulaceae	*Androsace umbellata*	Triterpenoid	Wang et al. (2008)
	Maesa ambigua	Triterpenoid	Foubert et al. (2009)
	Maesa argentea	Triterpenoid	Foubert et al. (2009)
	Maesa brevipaniculata	Triterpenoid	Foubert et al. (2009)
	Maesa perlarius	Triterpenoid	Foubert et al. (2009)
Ranunculaceae	*Actaea racemosa*	Triterpenoid	Cicek et al. (2010)
	Anemone raddeana	Triterpenoid	Lu et al. (2009)
	Clematis chinensis	Triterpenoid	Liu et al. (2009)
	Clematis parviloba	Triterpenoid	Yan et al. (2009)
	Nigella glandulifera	Triterpenoid	Xin et al. (2009)
	Pulsatilla turczaninovii	Triterpenoid	Xu et al. (2012)

Rhamnaceae	*Ampelozizyphus amazonicus*	Triterpenoid	Diniz et al. (2009)
Rosaceae	*Potentilla anserine*	Triterpenoid	Zhao et al. (2008)
	Sanguisorba officinalis	Triterpenoid	Zhang et al. (2012)
Rubiaceae	*Fadogia ancylantha*	Triterpenoid	Mencherini et al. (2010)
	Gardenia jasminoides	Triterpenoid	Wang et al. (2012)
Sapindaceae	*Blighia sapida*	Triterpenoid	Mazzola et al. (2011)
	Xanthoceras sorbifolia	Triterpenoid	Chan et al. (2008)
	Xerospermum noronhianum	Triterpenoid	Jean et al. (2009)
Sapotaceae	*Achras sapota*	Triterpenoid	Ahmed et al. (2008)
	Diploknema butyracea	Triterpenoid	Saha et al. (2010)
	Sideroxylon foetidissimum	Triterpenoid	Sanchez-Medina et al. (2009)
Scrophulariaceae	*Digitalis ciliata*	Steroidal	Gvazava and Kikoladze (2010)
Solanaceae	*Cestrum diurnum*	Steroidal	Fouad et al. (2008)
	Solanum lycocarpum	Steroidal	Nakamura et al. (2008)
Symplocaceae	*Symplocos lancifolia*	Triterpenoid	Acebey-Castellon et al. (2011)
Class of Liliopsida			
Agavaceae	*Agave utahensis*	Steroidal	Yokosuka and Mimaki (2009)
	Dracaena mannii	Steroidal	Tapondjou et al. (2008)
	Yucca desmettiana	Steroidal	Diab et al. (2012)
Asparagaceae	*Asparagus filicinus*	Steroidal	Wu et al. (2010)
Dioscoreacea	*Dioscorea nipponica*	Steroidal	Zhang et al. (2012)
Liliaceae	*Anemarrhena asphodeloides*	Steroidal	Lee et al. (2010)
	Chamaelirium luteum	Steroidal	Challinor et al. (2011)
	Chlorophytum borivilianum	Steroidal	Acharya et al. (2008)
	Chlorophytum nimonii	Steroidal	Lakshmi et al. (2009)
	Chlorophytum orchidastrum	Steroidal	Acharya et al. (2010)
	Paris delavayi	Steroidal	Zhang et al. (2009)
	Trillium erectum	Steroidal	Hayes et al. (2009)
	Tupistra chinensis	Steroidal	Liu et al. (2012)
	Ypsilandra thibetica	Steroidal	Xie et al. (2009)
Poaceae	*Panicum virgatum*	Steroidal	Lee et al. (2009)
Smilacaceae	*Smilax excelsa*	Steroidal	Ivanova et al. (2009)
Taccaceae	*Tacca integrifolia*	Steroidal	Shwe et al. (2010)

Sparg et al. (2004) have reported that steroidal saponins are almost exclusively found in the class of Liliopsida or monocotyledonous angiosperms. This is confirmed by the presence of steroidal saponin in Agavaceae, Asparagaceae, Dioscoreaceae, Liliaceae, Poaceae, Smilacaceae, and Taccaceae. The exception, however, found in Scrophulariaceae and Solanaceae, 2 plant families from class of Magnoliopsida or dicotyledoneus angiosperms in which all the species studied contained steroidal saponin. This diversity of

both triterpenoid and steroidal saponin sources is very important for future investigation, particularly as potential for various drug discovery programs.

4. The distribution and localization of saponins in plants

Saponin production has been found to vary in individual organs and tissues. Many plant species store saponins in the roots, where these molecules may act as antimicrobial phytoprotectants. For example, the major saponin from *Avena* spp., avenacin A-1, is localized in the epidermal cell layer of oat root tips and also in the lateral root initials, representing a chemical barrier to invading soil-borne microbes that attack plant tissue (Morrissey and Osbourn, 1999; Papadopoulou et al., 1999; Haralampidis et al., 2002).

Similar distribution of saponins was found in the roots of other species. The distribution of saikosaponins from *Bupleurum falcatum* roots has been reported in the outer phloem layer which contains many secretory canals, especially in the parenchyma cells located around the pericycle but not in the mucilaginous exudates within the secretory canals (Tani et al., 1986). In *Panax ginseng*, ginsenosides were found located outside the root cambium, particularly in the periderm and outer cortex peripheral to the phloem (Kubo et al., 1980; Tani et al., 1981).

The study of histochemical localization of saponins indicated that saponins react with vanillin-acetic acid glacial-perchloric acid solution to produce color from pale red to purplish red (Du and Liu, 1992; Tan et al., 2008). Based on this technique, histochemical studies of *Bupleurum chinense* organs show that in the root primary structures, saponins were mainly distributed in the pericycle and primary phloem. However, in the mature roots, they were mainly distributed in the vascular cambium and secondary phloem. Furthermore, during the vegetative and reproductive growth period, they were found to accumulate in mature fruit (Tan et al., 2008). The relationship between structural features of various organs and saponin accumulation was also studied in the important Chinese traditional medicinal species *Achyrantus bidentata* (Li and Hu, 2009). The results showed that leaves are an active site of synthesis rather than for storage. This is supported by the disappearance of saponins from leaves when they withered. Saponins are mainly accumulated in the root primary structure, as well as in the extra cambium cells and in the phloem cells of tertiary vascular bundles. In addition, the presence of saponin-related substances in stem vascular bundles indicates that the stem maybe essential as a transport

organ (Li and Hu, 2009). As mentioned by these authors, saponins are commonly found in the outer part especially in epidermal root tissue related to their function as an antimicrobial agent.

Triterpene saponin in *Polygala tenufolia* was found to be accumulated in roots, stems and leaves (Teng et al., 2009). In the roots, saponin accumulated mainly in phelloderm, secondary phloem, and rarely in xylem ray and xylem parenchyma cells. In stems, saponin was distributed in the cortex while less in the epidermis and secondary phloem. In leaves, saponin was mainly present in the palisade tissue and less in the epidermis and spongy tissue (Teng et al., 2009). This result indicates that saponin is mainly located in the parenchyma cells of the vegetative organ as similarly reported in the distribution of ginsenosides from *Gynostemma pentaphyllum* (Liu et al., 2005).

The accumulation of oligofurostanosides (steroidal saponins) has been reported in *Dioscorea caucasica* leaves. A histochemical study showed that this type of saponin accumulated in the special leaf epidermis receptacle cells. These cells are generally for storage of specific metabolites. On the other hand, oligofurostanosides have not been detected in the mesophyll tissues of the leaf where they are synthesized, which provides evidence for active transport from the mesophyll cells to the receptacle cells located in the epidermal layer (Gurielidze et al., 2004).

The quantification of triterpene saponins in aerial and subterranean organs of barrel medic *Medicago truncatula* revealed that roots contained the highest amounts of saponins followed by leaves and seeds, respectively. However, differential accumulation of specific triterpene saponins was reported. Medicagenic acid conjugates were highly accumulated in leaves and seeds, whereas soyasapogenol was found to be higher in the root (Huhman et al., 2005). Medicagenic acid conjugates in aerial parts have been correlated with biological activity in plant defense against herbivory (Agrell et al., 2003) and the accumulation of soyasapogenol in roots for plant-microbe signaling (Oleszek and Stochmal, 2002; Confalonieri et al., 2009) and allelopathic interactions (Waller et al., 1993; Khanh et al., 2005; Tava and Avato, 2006). In conclusion, the differential distribution of specific saponins is most likely the result of spatially controlled biosynthesis and active transport.

5. Saponins in plant defense

For many secondary metabolites, synthesis is stimulated upon challenge by biotic or abiotic stresses. Although this is a strategy to combat pathogens and build protection in a cost-effective way, some saponins are produced independently from external signals and contribute to the innate immunity. These saponins are referred to as phytoanticipins as they are present in unchallenged plants. The downside of accumulating saponins as a first defense is not only that it consumes substantial amounts of energy, but also that it allows pathogens to develop tolerance. This is avoided when saponin precursors accumulate and saponin content increases as the result of chemical modifications of precursor molecules, which are stimulated upon pathogen infection (Morrissey and Osbourn, 1999). The saponin content may also rise due to partial or complete hydrolysis of stored precursors as part of plant defense mechanism or by pathogen controlled degradation (Szakiel et al., 2011b).

Oats have become a model in which to study saponin biosynthesis as part of the interaction with fungi and bacteria (Osbourn, 1996; Osbourn et al., 2003; Mugford et al., 2009). Two types of saponins are constitutively produced, triterpene avenacins and steroidal avenacosides, which are present in roots and leaves respectively. Avenacins are available in an active glycoslyated form and accumulate in epidermal cells of the root tip. Avenacins have potent antifungal activity and confer resistance to a broad range of soil-borne pathogens. In addition, they are also released into the soil rhizosphere at biologically active concentrations by an unknown mechanism (Osbourn, 1996; Papadopoulou et al., 1999; Haralampidis et al., 2002). The active avenacin A-1 is stored in the vacuole and represents the major UV fluorescent compound allowing its detection in a fluorescence microscope (Papadopoulou et al., 1999). The increased disease susceptibility of oat variants lacking avenacins supports the importance of saponin in pathogen resistance as phytoprotectants. Furthermore, accumulation of the avenacin biosynthesis pathway intermediates in oat roots resulted in callose accumulation, a well known defense mechanism which suggests that accumulation of intermediates also triggers other defense mechanisms (Mylona et al., 2008).

In contrast to avenacins, steroidal avenacosides are known to be stored in vacuoles in an inactive bidesmosidic. They are activated when pathogenic fungi damage the plant tissue and disrupt membranes allowing the plant enzyme β-glucosidase to hydrolyse the D-

glucose unit forming toxic monodesmosides (Morant et al., 2008; Augustin et al., 2011). The active form of avenacosides then disrupts the plasma membrane of fungi by forming pores, which cause fungal cell death. Regardless of the exact mechanism, this deleterious effect is associated with the formation of complexes between saponin and ergosterol, the major membrane sterol in fungi, which is not present in plant cells (Weete, 1989; Bonanomi et al., 2009).

Another role of saponins in plants is to protect them against herbivores and/or insects. Plants frequently endure attack by life-threatening organisms which include herbivores and insects. The role of saponins in plant protection against herbivores is mostly based on their action as deterrents, toxins and digestibility inhibitors (Wittstock and Gershenzon, 2002; Massad, 2012; Mithofer and Boland, 2012). The exact mode of saponins as deterrents or toxins is not known, but it is mostly associated with their capacity to disrupt cell membranes. For instance, the lysis of hematocytes and a number of other cell types is a standard cytotoxicity test for saponins (Osbourn, 1996; Sparg et al., 2004). In insects, it was hypothesized that steroidal saponins may exert an antagonistic/competitive activity on the ecdysteroid receptor complex (EcR) based on their similarities to the insect moulting hormone 20-hydroxyecdysone (Dinan et al., 2001). However, De Geyter et al. (2012a) showed, using both triterpenoid and steroidal saponins on insect cells, that the action of saponins was not based on true antagonistic interaction with EcR signaling, but rather because of loss of cellular integrity presumably because of permeation of the insect cell membrane. In addition to cellular toxicity, saponins also showed deterrent or antifeedant activity against insects. It has been reported that a steroidal saponin, aginosid isolated from *Allium porrum* showed strong deterrent effects against two lepidopterans, the variegated cutworm (*Peridroma saucia*), and the bertha armyworm (*Mamestra configurata*) (Nawrot et al., 1991). Similarly, both deterrent and toxic effects were reported for triterpene saponins against aphids. Spraying faba bean leaves with *Quillaja saponaria* saponins at sublethal concentrations confirmed that aphids are more sensitive to the taste effect than to their toxicity (De Geyter et al., 2012b).

Constitutive metabolites can be produced in larger amounts after infection. In this circumstance, their status may refer to phytoalexin, although it would depend on whether or not the constitutive concentrations were sufficient for plant protection (Dixon, 2001). The inducibility of saponin biosynthesis has in most cases been analyzed using in vitro cultures.

For example, the exposure of plant cell cultures of *P. ginseng, Glycyrrhiza glabra*, *M. truncatula* to methyl jasmonate or other elicitors showed that squalene synthase, squalene epoxidase and β-amyrin synthase are rapidly induced (Hu et al., 2003; Hayashi et al., 2004; Suzuki et al., 2005; Lambert et al., 2011). However, the possible role of saponins as phytoalexin in intact plants is still unclear.

6. Saponin role in plant development

In addition to clear role of saponins in defense responses, they have also been demonstrated to play a role in plant growth regulation. For example, the study on germination of fenugreek seeds (*Trigonella foenum-graecum*) showed that the diffusible saponin substances located both in the endosperm and perisperm inhibited the production of α-galactosidase activity needed for germination (Zambou et al., 1993). In addition, a spirostanol saponin from *Yucca* induced callose synthesis in carrot cells (Messiaen et al., 1995).

A γ-pyronyl triterpenoid saponin termed chromosaponin 1 (CSI) was isolated from pea and other leguminous plants. CSI is a conjugate of soyasaponin I and γ-pyrone, and was reported to stimulate the growth of lettuce roots (*Lactuca sativa* L. ev. Grand Rapids). This stimulation was confirmed by a similar test using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), another amphipathic reductant. In contrast, no root regulation effects were observed using hydrophilic reductants including ascorbate, NADPH, NADH and glutathione (Tsurumi and Tsujino, 1995). Therefore, the root growth stimulatory activity was suggested to result from the amphipatic reducing activity of CSI.

The influence of CSI on root growth was further investigated in Arabidopsis (Rahman et al., 2001). CSI specifically interacts with the AUX1 protein in regulating the gravitropic response of Arabidopsis roots. Application of 60 μM CSI disrupted the vertically oriented elongation of wild type roots grown in agar plates but rescued the agravitropic phenotype mutant *aux1-7* roots. In *aux1-7* roots, CSI stimulated the uptake of IAA and induced gravitropic bending. The correlation between auxin uptake and gravitropic bending suggests that CSI may regulate the gravitropic response by inhibiting or stimulating the uptake of endogenous auxin in root cells (Rahman et al., 2001). Furthermore, the growth rate of Arabidopsis roots was accelerated when the seedlings were treated with 300 μM CSI. The

length of mature epidermal cells was increased by 2-3 folds and the number of epidermal cells was also increased by 2 fold (Rahman and Tsurumi, 2002). This result indicated that saponin affects cell elongation, which has been previously reported to be due to ethylene signaling inhibition (Rahman et al., 2000).

The expression pattern of saponin-related genes is very tightly regulated as shown in saponin studies in oat. In oat, the expression of avenacin A-1 s*aponin-deficient* (*sad*) mutants is restricted to the epidermal cell layer of root tips (Mylona et al., 2008). Mutants defective in *Sad1*, the gene for the first committed enzyme in the pathway, show normal root morphology. Thus, saponins are not required for root development. However, mutations at the two loci, *Sad3* and *Sad4* resulted in stunted root growth, membrane trafficking defects in the root epidermis, and root hair deficiency. *sad3* and *sad4* mutants are both affected in their ability to glucosylate avenacins and accumulate the same intermediate, monodeglucosyl avenacin A-1. These mutants have epidermis-specific membrane trafficking defects that are suppressed by mutations at *Sad1*, indicating that accumulation of monodeglucosyl avenacin A-1 is the cause rather than a consequence of the observed root defects. This result suggested that *Sad3* and/or *Sad4* may be required specifically for triterpene glycosylation. Alternatively, they may have functions in root growth and development that indirectly affect the glycosylation process.

Soyasapogenol B, a triterpene saponin which is present in most legume species was reported to stimulate germination of barley seeds (*Hordeum vulgare*) (Macias et al., 1997). In addition, Soyasapogenol B isolated from common vetch (*Vicia sativa*) showed a stimulatory activity on broomrape (*Orobanche minor*) seed germination, but did not stimulate any other *Orobanche* species (Evidente et al., 2011). It showed high stimulatory activity on *O. minor* seed germination when tested at 10^{-3} - 10^{-4} M. At 10^{-5} M, no stimulatory activity was observed, suggesting poor specificity. Nevertheless, this stimulatory activity could be important for weed germination control strategies, as an alternative to strigolactones which are unstable in water (Akiyama et al., 2010).

Expression and biochemical studies have suggested a possible involvement of β-amyrin and derived saponins in the regulation of root nodulation. Indeed, over-expression of *AsOXA1*, a β-amyrin synthase from *Aster sedifolius* in *Medicago truncatula* caused in a subset of lines enhanced root nodulation (Confalonieri et al., 2009). The improvement of nodulation is however presumably indirect as these plants did not exhibit significant

differences in saponin content in roots or in growth and biomass performance compared with the control plants. Similarly reported by Hayashi et al. (2004) who examined the expression pattern of β-amyrin synthase in different tissues of *Glycyrrhiza glabra*, found high levels of β-amyrin synthase mRNA and triterpene saponins in root nodules.

Lupeol, another type of the triterpene aglycon, has been reported to influence nodule formation in *Lotus japonicus* by down-regulating the expression of early nodulin gene *ENOD40*. The silencing of lupeol synthase (LuS) gene resulted in a phenotype that was similar to that observed for plants overexpressing *ENOD40* (Delis et al., 2011). Thus, in the absence of lupeol, the expression of *ENOD40* is elevated leading to a more rapid nodulation phenotype. As previously reported, *ENOD40* leads to accelerated nodulation in *Medicago* as a consequence of extensive cortical cell division and increased initiation of primordia (Charon et al., 1999).

Takagi and co-workers introduced two different RNAi cassettes driven by seed-specific promoter to target β-amyrin synthase expression in *Glycine max* (Takagi et al., 2011). This experiment resulted in a sufficient reduction of saponin content of soybean seeds. Such saponin deficiency however, did not appear to affect the growth of plants under greenhouse conditions, suggesting that seed saponins are not required for normal growth and development in soybean (Takagi et al., 2011).

7. Concluding remarks

The wide spread occurrence of plant saponins underwrites the importance of this group of secondary metabolites in the plant kingdom. While most studies support a role for saponins as phytoprotectants, a role for saponins in the regulation of plant growth and development remains one of the possible functions that warrant further investigation.

The biosynthetic pathway of saponin is still not fully unraveled although major progress has been made for oat and other saponin producing plants. Furthermore, the diversity of both triterpenoid and steroidal saponins has lead to increasing interest of these compounds in recent years. It also offers the opportunity for further research on phytochemistry and biological activity of saponins. Accordingly, much effort has been put into elucidation of possible roles of saponins in plants, as well as their prospect application for industry. In the next years additional efforts should focus on the tissue specificity of saponin production as well as more detailed analysis of the cellular localization and storage

of saponins. These studies will provide further insight into the functions of saponins in plants and promote the exploration of plant-derived natural products in the future.

Acknowledgement

The authors would like to thank the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia and the BOF (special research fund No. 01WI0111, Ghent University) for A.F. PhD fellowship.

2 | In vitro propagation of four saponin producing *Maesa* species

Cover art: In vitro-cultivated *Maesa lanceolata*

2

In vitro propagation of four saponin producing *Maesa* species

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Parts of the manuscript have been published in:

Ahmad Faizal, Ellen Lambert, Kenn Foubert, Sandra Apers, and Danny Geelen. **In vitro propagation of four saponin producing *Maesa* species**. *Plant Cell Tissue Organ Culture* (2011), 106:215-223

* Author contributions: Adventitious shoot regeneration, plant cultivation, flow cytometry, statistical analysis, and writing of the chapter.

Abstract

A successful micropropagation system was developed for four different medicinal *Maesa* species. Multiple shoots were induced through both axillary bud formation and adventitious shoot regeneration from leaf explants. The explants were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA), thidiazuron (TDZ) and/or α-naphthalene acetic acid (NAA). The success of regeneration varied for different species and depended on the type and concentration of plant growth regulators. Regenerated shoots spontaneously developed roots within 6 weeks on MS hormone-free medium. The rooted shoots were transferred to the greenhouse with a 100% success rate. Furthermore, flow cytometry analysis indicated that there were no changes in ploidy level of those regenerated shoots as compared with wild type adult plants. Therefore, the protocol also provides an effective means for the in vitro conservation of *Maesa* spp. that produce pharmaceutically interesting saponins.

1. Introduction

Genus *Maesa* comprises approximately 150 species, from small trees and shrubs to woody vines from rainforest canopies. They occur primarily in the tropics of Africa and Asia. Traditionally, *Maesa* was placed in the family of Myrsinaceae (order of Ericales). However, the family in which *Maesa* has been placed has changed due to different interpretations of family limits within this group, especially in floral characteristics (Caris et al., 2000). Because of these differences, Anderberg and co-workers suggested that the genus *Maesa* should be split from the Myrsinaceae and placed in a newly defined family, Maesaceae, more closely related to Primulaceae (Anderberg et al., 2000). Most recently, genus *Maesa* is placed in an enlarged Primulaceae (Bremer et al., 2009; Utteridge, 2012).

Generally, *Maesa* plants flower early in the year, however, the flowering period is strongly affected by climate conditions. Study of floral development and breeding of *Maesa japonica* and *M. perlarius* showed that both species are functionally dioecious. This means that the species have two sexual phenotypes differing in both primary and secondary sexual characteristics (Fig. 1). The consequence is that there are plants with a functional pistillate will set fruit and plants with a functional staminate will not set fruit (Utteridge and Saunders, 2001). It appears likely, however, that many species of *Maesa* show a degree of sexual dimorphism with associated functional dioecy (Ma and Saunders, 2003).

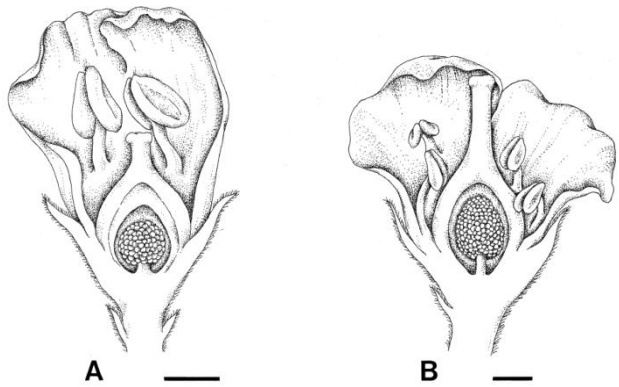

Fig. 1 Half-flower illustration of *M. perlarius* showing two sexual phenotypes. A Staminate flower. B Pistillate flower. Scale bars = 500 µm (Ma and Saunders, 2003).

Four *Maesa* species that were used in this research include *Maesa argentea, M. balansae, M. lanceolata,* and *M. perlarius* which will now be described. *M. argentea* Wall is a shrub or rarely small trees to 5 m tall. The plants grow in broad-leaved forests, hilly areas,

valleys, stream banks and damp places of many Asian countries (Shan, electronic flora of China). *M. balansae* Mez. is a glabrous shrub (1-3 m tall) mainly found in Vietnam and China. *M. perlarius* (Lour.) Merr. is a small shrub of 1 to 3 m tall and is found in broad-leaved forest, shrubby areas, hillsides and damp places in Thailand, Vietnam, Taiwan, and China. *M. lanceolata* Forssk. occurs primarily in central and south-east Africa. They can be straggling shrubs of 2 to 3 m tall or small trees up to 9 m. They grow also on cliff tops as in midland or coastal areas.

All four species play an important role in traditional medicine and ancient practices. Investigation of the methanol extract of dried leaves of different *Maesa* species resulted in the identification of triterpenoid saponin mixtures. In vitro bioactivity assays showed that *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* have virucidal, molluscicidal, cytotoxic, haemolytic and anti-leishmanial actions (Sindambiwe et al., 1996; Foubert et al., 2009). More extensive studies on *M. balansae* saponins have shown that these metabolites are effective in curing visceral leishmaniasis in mice and hamsters. The potency of the saponins is comparable to that of the commercial drug against leishmaniasis, liposomal amphotericin B (Maes et al., 2004).

There are, however, difficulties accompanied with conventional propagation of *Maesa* species, e.g. the plants are rarely flowering in greenhouse conditions. Therefore, in vitro tissue culture techniques have become a feasible alternative to improve the efficiency of propagation as well as to facilitate some in vitro experiments such as genetic transformation, protoplast fusion and investigation of the gene expression in saponin biosynthesis. In fact, in vitro culture has already been shown to be an efficient method for propagating medicinal plants (see reviews by Rout et al. (2000) and Debnath et al. (2006)). Many plants are grown under in vitro conditions to conserve the germplasm, examples are micropropagation of *Asparagus racemosus* (Bopana and Saxena, 2008), *Searsia dentata* (Prakash and Van Staden, 2008), *Ceropegia intermedia* (Karuppusamy et al., 2009), *Cecropia* spp. (Nicasio-Torres et al., 2009), and *Bacopa monnieri* (Singh et al., 2009). In some cases, tissue and cell culture is also used for the production of interesting secondary metabolites. In vitro root cultures of *Catharanthus roseus* are used for the production of two anti-cancer alkaloids, vinblastine and vincristine (Ataei-Azimi et al., 2008). Cell cultures of *Taxus* spp. are used to produce large amounts of alkaloid taxol, which is a chemotherapeutic agent, approved in the treatment of a variety of cancers. Taxol is currently supplied through both a

semi-synthetic process and plant cell culture (Vongpaseuth and Roberts, 2007). In addition, cell cultures of *Panax ginseng* are used for the commercial production of triterpene saponins (Wu and Zhong, 1999).

We are investigating the biosynthesis and production of the saponins in four different medicinal *Maesa* species. In this study, we present the optimization of an in vitro propagation method that greatly facilitates the production of genetically stable *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* plantlets that show vigorous growth in the greenhouse.

2. Results and discussion

Micropropagation through axillary shoot formation

The experiments conducted to optimize the medium for multiplication of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* are summarized in Table 1. The effect of different hormones on the induction of shoots was clearly dependent on the species used. For *M. argentea* and *M. lanceolata* the highest number of shoots, 6.7 and 6.3 respectively, was achieved when using 6-benzyladenine (BA) alone at a concentration of 13.2 µM. *M. balansae* responded best to 8.8 µM BA with 2.7 shoots per explant. Other hormone treatments did not show a significant difference with the control. For *M. perlarius* none of the hormone treatments stimulated shoot induction and no difference with the hormone free control medium was observed. Noticeably, the multiplication rate of the control plants of *M. perlarius* was higher than that of the controls of the other species.

For *M. argentea, M. balansae* and *M. lanceolata* the number of shoots per explant was always highest when treated with BA alone. Addition of α-naphthaleneacetic acid (NAA) had a negative effect on the number of shoots formed. This is in contrast with the results published for *Maesa ramentacea*, where a synergistic action between BA and NAA was observed (Kanchanapoom and Boonvanno, 2000). It was also noted that for *M. argentea, M. lanceolata* and *M. perlarius* the highest concentration of BA induced highest number of shoots; therefore it is possible that concentrations higher than 13.2 µM of BA could be even more effective. Though, it should be considered that high concentrations of BA can be toxic to the plants through induction of programmed cell death (Carimi et al., 2004).

Table 1 Influence of the plant growth regulators BA and NAA on axillary shoot multiplication in different *Maesa* species 8 weeks after incubation on multiplication medium

PGRs (µM)		No. of shoots/explant (± SEM)			
BA	NAA	*M. argentea*	*M. balansae*	*M. lanceolata*	*M. perlarius*
-	-	1.0 ± 0.0^{d}	1.4 ± 0.3^{bc}	1.3 ± 0.2^{c}	2.2 ± 0.8^{abc}
4.4	-	3.0 ± 0.4^{bc}	2.2 ± 0.2^{ab}	1.7 ± 0.3^{c}	2.7 ± 0.5^{ab}
8.8	-	4.7 ± 0.3^{b}	2.7 ± 0.2^{a}	4.7 ± 0.7ab	2.8 ± 1.9^{bc}
13.2	-	6.7 ± 1.1^{a}	1.8 ± 0.5^{abc}	6.3 ± 1.2^{a}	3.2 ± 0.4^{a}
4.4	10.7	0.7 ± 0.2^{d}	1.3 ± 0.2^{bc}	0.8 ± 0.3^{c}	0.5 ± 0.2^{c}
13.2	13.5	0.0 ± 0.0^{d}	1.0 ± 0.0^{c}	1.2 ± 0.2^{c}	0.3 ± 0.2^{c}
22.2	5	1.5 ± 0.2^{cd}	2.0 ± 0.6^{abc}	2.8 ± 0.3^{bc}	3.8 ± 0.9^{a}

Different letters indicate significant differences ($P < 0.05$) within one species according to Duncan test.

Adventitious shoot induction

To obtain adventitious shoot induction, leaf explants were incubated on MS medium supplemented with various concentrations of either BA or thidiazuron (TDZ), individually or in combination with NAA. For this experiment, two different types of cytokinins were chosen; BA is a purine-type cytokinin, while TDZ is a phenylurea with cytokinin like actions. Adventitious shoots were observed for all four *Maesa* species; however, optimal shoot induction conditions differed for the analyzed species (Table 2). Generally, small outgrowths were induced at the surface of leaf explants after 4 to 5 weeks of culture. No shoots were induced on hormone-free medium or when cytokinins alone were added to the culture medium, implying that a combination of cytokinin and auxin was necessary for adventitious shoot formation in *Maesa* leaf explants. The only exception is shoot induction on *M. argentea* leaves with the highest concentration of TDZ.

Application of BA in combination with NAA resulted in very low frequency shoot formation for two out of the four *Maesa* species. For *M. argentea*, shoot induction was only achieved with 22.2 µM BA and 1.35 µM NAA (0.2 shoots per explant). For *M. perlarius* two combinations of BA and NAA, namely 13.3 µM BA with 1.35 µM NAA and 22.2 µM BA with 2 µM NAA induced adventitious shoots, with a mean of 2.3 and 0.3 shoots per explant, respectively. For all four *Maesa* species, treatment of leaves with BA in combination with NAA often lead to root induction, which was not observed when BA alone was used.

In contrast to the results of shoot induction using BA in combination with NAA, the combination of TDZ and NAA induced multiple adventitious shoots on all four *Maesa* species. This observation is suggestive for a synergetic effect of TDZ and NAA. For *M. argentea*, the

highest average number of shoots (9.7 shoots per explant) was obtained when leaf explants were incubated on MS medium supplemented by 13.6 µM TDZ and 2 µM NAA (Fig. 2a). Except for the two lower concentrations of TDZ alone (4.5 and 13.6 µM), all the combinations and concentrations of TDZ and NAA resulted in shoot induction for *M. argentea*. For the other species shoots were also observed, however, not so frequently as for *M. argentea*. *M. lanceolata* leaves showed shoot induction with three combinations of TDZ and NAA, with a maximum of 4.1 shoots per explant when 22.7 µM TDZ was combined with 1.35 µM NAA (Fig. 2c). *M. perlarius* leaf explants developed adventitious shoots with only one combination, 4.5 µM TDZ and 0.5 µM NAA, with a mean of 3.6 adventitious shoots per explant (Fig. 2d). *M. balansae* showed a lower frequency of shoots regeneration with at maximum 1.2 shoots per explant when the highest concentrations of TDZ and NAA were combined (22.7 µM and 2 µM, respectively) (Fig. 2b). A combination of 4.5 µM TDZ and 0.5 µM NAA also induced shoots, however, at a very low number (0.6 shoots per explant). Except for *M. argentea*, all species showed root formation in addition to shoot formation.

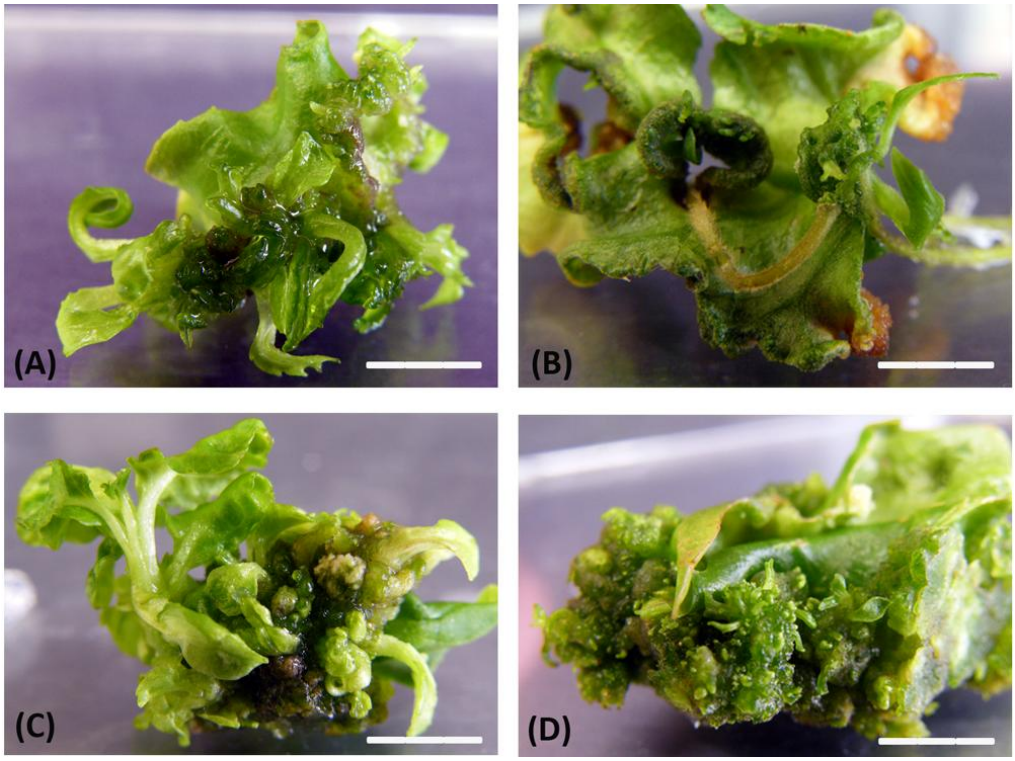

Fig. 2 Induction of adventitious shoots on leaf explants of (**a**) *M. argentea*, (**b**) *M. balansae*, (**c**) *M. lanceolata* and (**d**) *M. perlarius*. Pictures were taken 8 weeks after culture on MS medium supplemented with TDZ and NAA. *Bar* = 0.5 cm.

Taken together, these results suggest that TDZ acts synergistically with NAA to promote shoot induction. In contrast to addition of BA and NAA, TDZ played an essential role in inducing adventitious shoot induction on leaf explants from *Maesa in vitro* plants and proved to be the more effective cytokinin in our study. The higher effectiveness of TDZ as compared to BA, for induction of adventitious shoots from leaf explants, was also reported for other plant species such as *Echinacea purpurea* (Jones et al., 2007), *Paulownia tomentosa* (Corredoira et al., 2008), *Lysimachia* spp. (Zheng et al., 2009) and *Mimulus aurantiacus* (Murovec et al., 2010). Thidiazuron, a synthetic phenylurea derivative, is one of the most active cytokinin-like compounds for woody plant tissue culture (Huetteman and Preece, 1993; Lu, 1993). Unlike classic cytokinins, TDZ is competent of fulfilling both the cytokinin and auxin requirement of various regenerative responses of many different plant species (Jones et al., 2007). A low concentration of NAA was necessary to induce direct shoot regeneration from leaf explants. This means that NAA can be considered as a critical growth regulator for shoot regeneration of *Maesa* spp. De Gyves and coworkers hypothesized that there is a synergism existing between TDZ and both endogenous and exogenous auxin (De Gyves et al., 2001). This finding corresponds with our results that in general the combination of TDZ and NAA promoted more shoots compare to application of TDZ alone. The combination of TDZ and NAA has also been reported to induce shoot regeneration from leaf explants of several plant species (Espinosa et al., 2006; Feng et al., 2010; Zhou et al., 2010).

Table 2 Effect of the plant growth regulators BA, TDZ and NAA on adventitious shoot regeneration from mature leaf explants of 4 *Maesa* spp.

Plant growth regulators (µM)			*M. argentea*		*M. balansae*		*M. lanceolata*		*M. perlarius*	
BA	TDZ	NAA	No. of shoots/explant (± SEM)	Rooting	No. of shoots/explant (± SEM)	Rooting	No. of shoots/explant (± SEM)	Rooting	No. of shoots/explant (± SEM)	Rooting
-	-	-	0.0 ^{d}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	no
4.4	-	-	0.0 ^{d}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	no
13.3	-	-	0.0 ^{d}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	no
22.2	-	-	0.0 ^{d}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	no
4.4	-	0.5	0.0 ^{d}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	yes
4.4	-	1.4	0.0 ^{d}	no	0.0 ^{d}	yes	0.0 ^{b}	yes	0.0 ^{c}	yes
4.4	-	2	0.0 ^{d}	no	0.0 ^{d}	yes	0.0 ^{b}	yes	0.0 ^{c}	yes
13.3	-	0.5	0.0 ^{d}	no	0.0 ^{d}	yes	0.0 ^{b}	no	0.0 ^{c}	yes
13.3	-	1.4	0.0 ^{d}	yes	0.0 ^{d}	yes	0.0 ^{b}	yes	**2.3 ± 0.9** ^{b}	yes
13.3	-	2	0.0 ^{d}	yes	0.0 ^{d}	yes	0.0 ^{b}	yes	0.0 ^{c}	yes
22.2	-	0.5	0.0 ^{d}	no	0.0 ^{d}	yes	0.0 ^{b}	no	0.0 ^{c}	no
22.2	-	1.4	0.2 ± 0.22 ^{d}	yes	0.0 ^{d}	yes	0.0 ^{b}	no	0.0 ^{c}	no
22.2	-	2	0.0 ^{d}	yes	0.0 ^{d}	yes	0.0 ^{b}	yes	0.3 ± 0.3 ^{c}	no
-	4.5	-	0.0 ^{d}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	no
-	13.6	-	0.0 ^{d}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	no
-	22.7	-	1.7 ± 0.9 ^{cd}	no	0.0 ^{d}	no	0.0 ^{b}	no	0.0 ^{c}	no
-	4.5	0.5	0.4 ± 0.3 ^{d}	no	0.6 ± 0.34 ^{d}	yes	0.0 ^{b}	no	3.6 ± 0.6 ^{b}	no
-	4.5	1.4	4.9 ± 2.9 ^{b}	no	0.0 ^{d}	yes	0.0 ^{b}	no	0.0 ^{c}	no
-	4.5	2	0.4 ± 0.4 ^{d}	no	0.0 ^{d}	yes	0.0 ^{b}	yes	0.0 ^{c}	yes
-	13.6	0.5	0.7 ± 0.4 ^{d}	no	0.0 ^{d}	yes	0.0 ^{b}	no	0.0 ^{c}	no
-	13.6	1.4	5.0 ± 1.2 ^{b}	no	0.0 ^{d}	yes	1.3 ± 0.6 ^{ab}	no	0.0 ^{c}	no
-	13.6	2	**9.7 ± 2.1** ^{a}	no	0.0 ^{d}	yes	0.0 ^{b}	yes	0.0 ^{c}	yes
-	22.7	0.5	5.4 ± 0.9 ^{b}	no	0.0 ^{d}	yes	0.0 ^{b}	no	0.0 ^{c}	no
-	22.7	1.4	2.6 ± 0.9 ^{cd}	no	0.0 ^{d}	yes	**4.1 ± 1.6** ^{a}	no	0.0 ^{c}	no
-	22.7	2	1.6 ± 0.8 ^{cd}	no	**1.2 ± 1.2** ^{cd}	yes	1.9 ± 1.2 ^{ab}	yes	0.0 ^{c}	Yes

Shoot induction was evaluated and scored after 8 weeks; for each species, best result is indicated in bold. Different letters indicate significant differences ($P < 0.05$) within one species according to Duncan test.

Rooting and acclimatization

Regenerated shoots elongated and developed roots in basal MS media without phytohormones with an efficiency of 100%. Generally, *Maesa* roots started to emerge within 2 weeks. Plantlets spontaneously produced well developed root system within 6 weeks on hormone-free medium (Fig. 3a-d). The very efficient rooting mechanism of these plants provides an additional advantage for the rapid clonal propagation without any growth regulators needed. This spontaneous root formation is effective during the establishment of the plantlets in soil as well. Rooting of shoots without any addition of hormones is also reported for *Aloe polyphylla* (Bairu et al., 2007), *Drymaria cordata* (Ghimire et al., 2010), *Tuberaria major* (Gonçalves et al., 2010), and *Vitis champinii* (Mukherjee et al., 2010). However, most species require auxin treatment for root induction prior to acclimatization.

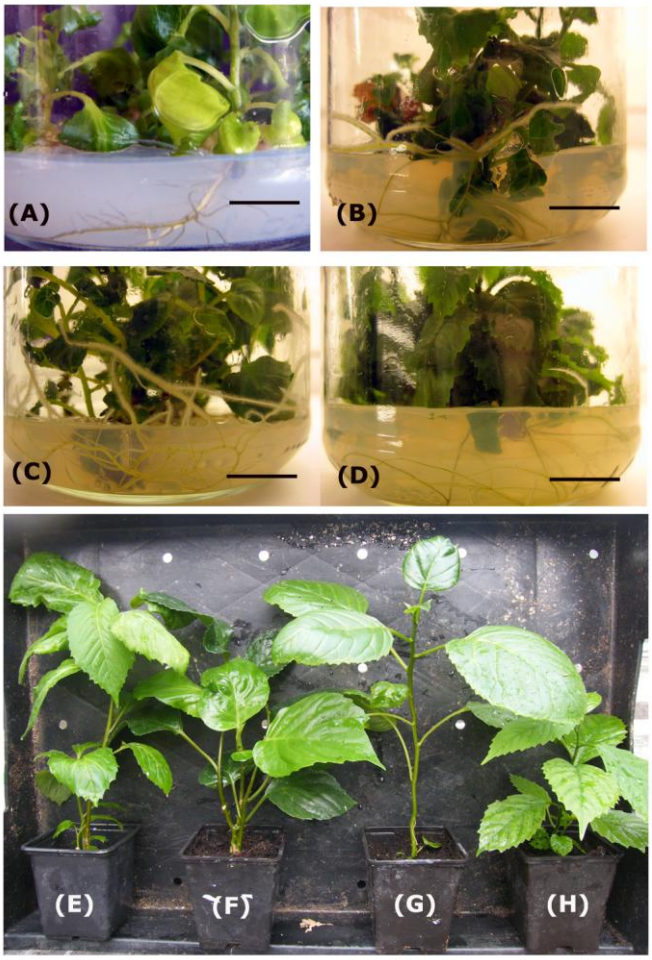

Fig. 3 Rooting of *in vitro* regenerated shoots of (**a**) *M. argentea*, (**b**) *M. balansae*, (**c**) *M. lanceolata* and (**d**) *M. perlarius*. Root pictures were taken 8 weeks after culture on MS basal medium. Regenerated shoot acclimatized for 4 months in greenhouse conditions of (**e**) *M. argentea*, (**f**) *M. balansae*, (**g**) *M. lanceolata* and (**h**) *M. perlarius*. *Bar* = 1 cm.

For acclimatization, regenerated and rooted shoots from all explants of *Maesa* spp. could be hardened with a 100% efficiency rate. After transfer to greenhouse condition, the plantlets continued to grow and developed into normal and vigorous plants (Fig. 3e-h).

Stability of regenerated shoots at ploidy level

Inducing adventitious shoots through *in vitro* regeneration is sometimes accompanied by genetic instability through a process known as somaclonal variation (Larkin and Scowcroft, 1981). To further investigate the impact of tissue culturing on the plant genetic stability, shoots regenerated through the application of different types and concentrations of plant growth regulators and from different types of explants were analyzed. Since this regeneration process escapes the normal plant fertilization and development, it is also possible that the variation occurs by epigenetic factors (Kaeppler et al., 2000). The variability that is commonly noticed are the ploidy level, chromosome structure, mitotic abnormalities and other cytological disorders (Radić et al., 2005). Flow cytometry is considered as a powerful tool for estimating DNA ploidy level in plant in vitro (Cousin et al., 2009) and has already been used to investigate the genetic variability of in vitro regenerated shoots (Mallón et al., 2010; Obae and West, 2010; Vujovic et al., 2010).

The ploidy level of *Maesa* regenerated shoots was determined through analyzing small pieces of leaves. The relative nuclear DNA content of *Maesa* spp. was determined for different plant categories: greenhouse plants grown from seedling, plantlets grown from axillary buds and adventitious shoots from leaf explants. The flow cytometry analysis showed the similar peaks in all samples within every *Maesa* species (Fig. 4). No significant differences in DNA content were observed for each of the in vitro cultivated *Maesa* plant material indicating that no changes in ploidy level occurred during the regeneration process. In addition, early stage of regeneration process from leaf explants stimulated by hormones was also analyzed to check for ploidy level changes. Also in this plant material we did not observe deviations in DNA content, indicating that the hormone treatment did not alter the ploidy level of the plants.

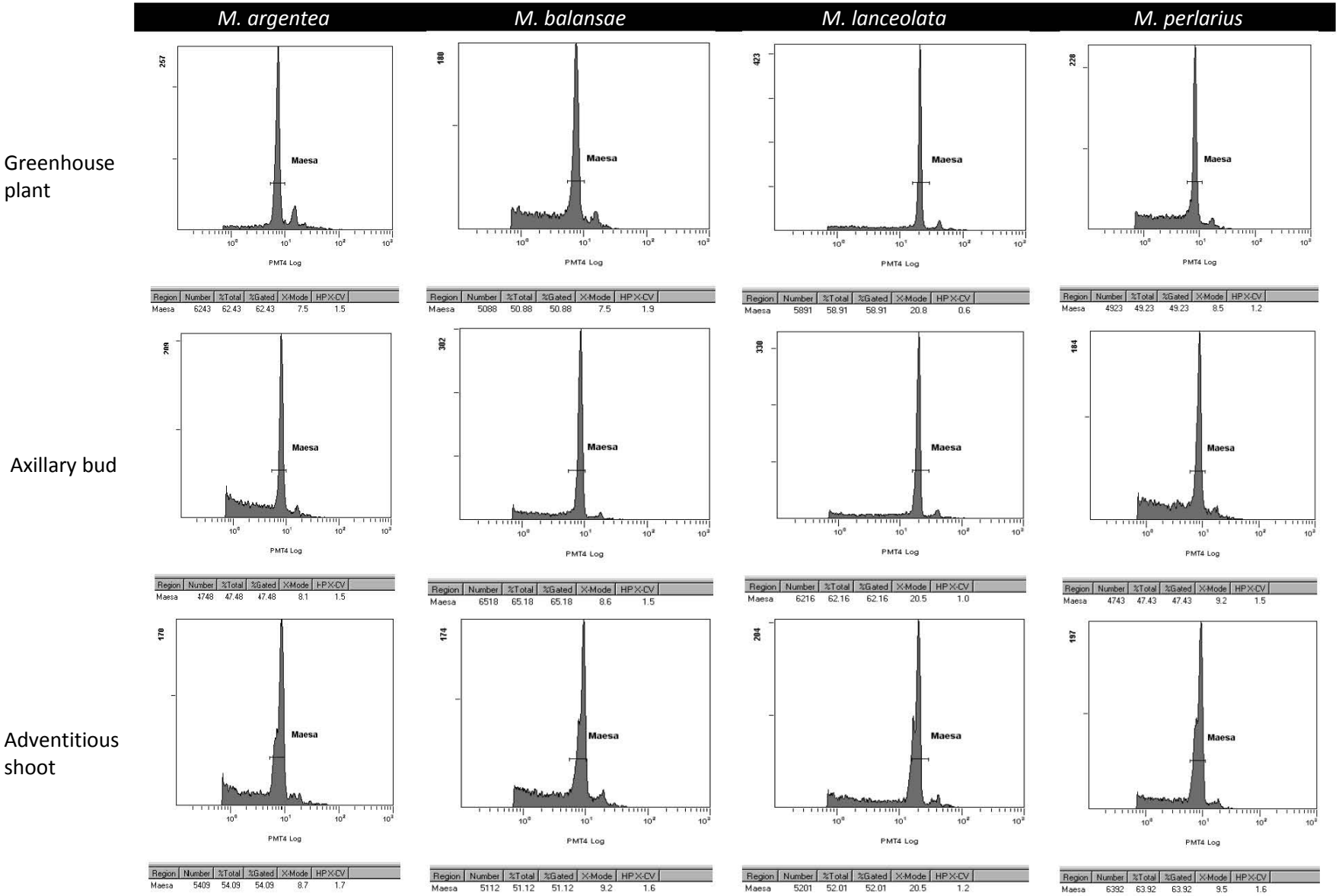

Fig. 4 Histogram of relative fluorescence intensity (log-transformed, PMT4 log) of isolated nuclei from 4 *Maesa* species.

In conclusion, this report provides an efficient and reproducible protocol for adventitious and axillary shoot regeneration of the species *M. argentea, M. balansae, M. lanceolata* and *M. perlarius*. The regenerated plants are of the same ploidy as the parental material. The established protocol will be useful not only for the micropropagation and conservation of *Maesa* spp., but also as a potential system for genetic modification of these medicinal plants.

3. Materials and methods

Plant Material

M. lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). *M. balansae* and *M. perlarius* seeds were collected in the Trang Dinh district, Vietnam by Nguyen Tap (National Institute of Medicinal Materials Lang Son Province). Finally, *M. argentea* seeds (no. 61-2068) were provided by the National Botanical garden (Meise, Belgium). The seeds were rinsed in 70% (v/v) ethanol for 30 seconds and subsequently surface sterilized with a 70% (v/v) solution of a commercial disinfection product (Haz-tabs; Guest Medical, Kent, UK). After three washes with distilled water, the seeds were placed on MS basal medium (Murashige and Skoog, 1962) supplemented with 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham) and 3% (w/v) sucrose (with pH 5.8). Seeds were germinated in a 16/8 h light/dark photoperiod at 26°C.

Axillary shoot formation

Three month old seedlings of *M. argentea, M. balansae, M. lanceolata and M. perlarius* were used as explant source for micropropagation through an axillary branching method. Shoots were excised, defoliated and implanted vertically into MS basal medium supplemented with 3% (w/v) sucrose and 0.15% (w/v) Gelrite (Marck & Co., Kelco Division, USA). For shoot multiplication, the basal medium was supplemented with BA and NAA at different concentrations. The following concentrations were used: 4.4 µM, 8.8 µM, 13.2 µM and 22.2 µM BA, alone or in combination with 5 µM, 10.7 µM and 13.5 µM NAA. Shoots were incubated at 26 °C

in a 16/8 h light/dark period and axillary shoot formation was evaluated 8 weeks later.

Adventitious shoot induction

Fully developed leaves from in vitro grown plants of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* were isolated and used as explants. Leaves (petiole was cut off from the leaf bases) were placed with the adaxial side in contact with MS basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. For shoot induction, auxin and cytokinins were added to the basal medium in different concentrations, either alone or in combinations. The following growth regulators were used in the given concentrations; 0.5 µM, 1.4 µM and 2 µM NAA; 4.4 µM, 13.3 µM and 22.2 µM BA and 4.5 µM, 13.6 µM and 22.7 µM TDZ. Leaves were incubated at 26 °C with 16/8 h light/dark photoperiod. The average number of adventitious shoot induced per explant was recorded after 8 weeks of culture.

Rooting and acclimatization

Multiple shoots were formed through axillary branching and adventitious shoot induction. These shoots were isolated and transferred to basal MS medium lacking growth regulators and supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar, for elongation and rooting in one single step. Shoots were incubated in a 16/8 h light/dark photoperiod at 25 °C.

Rooted plantlets were gently and thoroughly washed with water, to remove attached medium from the roots, and were transferred to 9 x 9 cm^2 small plastic pots containing a mixture of sand and peat soil (1:1). The plantlets were placed in a small greenhouse with a high humidity for 3 weeks to gradually acclimatize to greenhouse conditions.

Flow cytometry

Flow cytometry was performed to check ploidy of the regenerated shoots. Leaf samples derived from both micropropagation through axillary branching and adventitious shoot from leaf explants were compared with wild adult plants. Approximately 50-100 mg of both young *in vitro* and wild adult plant leaf material

was chopped with a razor blade in 2 mL Galbraith buffer (45 mM $MgCl_2$, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 30 mM sodium citrate and 0.1% (v/v) Triton X-100) (Galbraith et al., 1983; Loureiro et al., 2006) to isolate nuclei. 5% (w/v) of Polyvinylpyrrolidone 10000 (PVP-10) was also added to the extraction buffer to neutralize interference of cell metabolites in the measurements. The nuclei suspension was filtered through a 50 µm strainer to remove debris. Nuclei were stained by mixing 400 µL of nuclei suspension with 50 µL propidium iodide (0.5 mg/mL). 50 µg/mL RNAse was added to prevent staining of double-stranded RNA. The DNA content of the isolated samples was measured by using a Beckman Coulter EPICS® Altra™ Flow Cytometer. The instrument was equipped with a 15 mW 488 nm air-cooled argon-ion laser. Fluorescence was detected through a 575 nm band-pass filter. Disintegrated nuclei and other cell debris signals were eliminated from analysis by two gating systems; forward scatter based on nuclei proportional and PMT3 based on propidium iodide fluorescence. The gates were consistently maintained for all samples in each run and the resulting PMT4 histograms were analyzed using EXPO™32 MultiCOMP software (Beckmann Coulter). Together with each leaf sample, leaf tissue from diploid (2n) *Arabidopsis thaliana* was included as an external reference standard.

Statistical analysis

Each petri dish and bottle with four or five explants was considered as one replicate. The presented experiments are comprised of at least three replicates. Number of shoots was recorded 8 weeks after induction both for axillary shoot formation and adventitious shoot from leaf explants. The data were analyzed by one-way ANOVA followed by Duncan test ($P < 0.05$).

Acknowledgements

We thank the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia and the BOF (special research fund No. 01WI0111, Ghent University) for A.F. PhD fellowship.

3 | Studies on saponin production in *Maesa* species

Cover art: *Dream bubble* by Shunkoba | www.flickr.com/photos/shunkoba

3

Studies on saponin production in *Maesa* species

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Kenn Foubert[3], Sandra Apers and Danny Geelen

Authors' contributions:
[1] In vivo and in vitro plant cultivation, metabolite extraction, TLC analysis, data analysis and writing chapter.
[2] In vitro elicitation and TLC analysis.
[3] HPLC-MS analysis.

Abstract

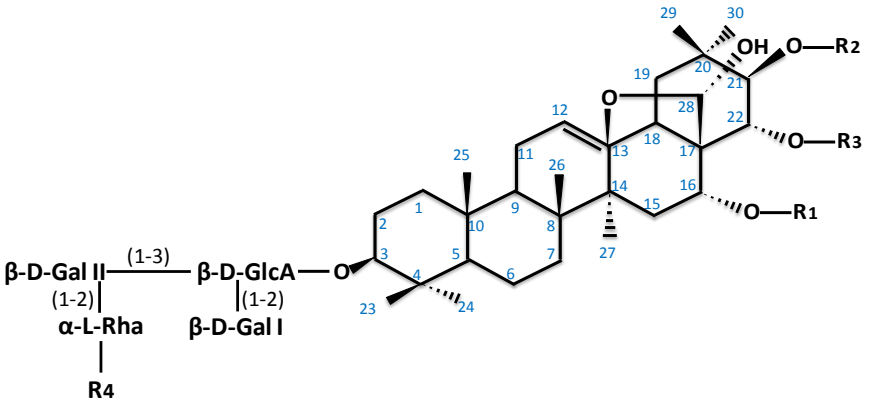
The continuous need for new compounds with important medicinal activities has lead to the identification and characterization of various plant-derived natural products. As a part of this program, we studied the saponin production from four medicinal plants *Maesa argentea*, *M. balansae, M. lanceolata* and *M. perlarius* and evaluated several treatments to enhance their saponin production. In this chapter, we present the analyses of saponin production from greenhouse grown plants by means of TLC and HPLC-MS. We observed that the content of saponin from these plants varied depending on organ and physiological age of the plants. In addition, we established in vitro tissue cultures to facilitate the modulation of culturing conditions and enhancement of saponin production. The impact of plant growth regulators and elicitors on saponin accumulation was analyzed using TLC. The production of saponin was very stable and not affected by treatment with auxin, giberellic acid, abscisic acid, ethylene, methyl jasmonate, and salicylic acid. In conclusion, *Maesa* saponins are constitutively produced in plants and the level of these compounds in plants is mainly affected by the developmental or physiological stage.

1. Introduction

The first report on triterpenoid saponins in *Maesa lanceolata* was published by Sindambiwe and co-workers within the framework of research on natural anti-infectious agents (Sindambiwe et al., 1996). Using a bioassay-guided fractionation, a saponin mixture was isolated from the leaves of *M. lanceolata*. Within the mixture, six homologous triterpene saponins were identified using two dimensional nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) and by comparison with known saponins containing a related glycosidic moiety (Sindambiwe et al., 1996). Subsequently, an effort was undertaken to obtain pure saponins via a semipreparative scale using wide pore reverse-phase high performance liquid chromatography (HPLC). In this way, 14 pure triterpenoid saponins were isolated (Apers et al., 1998). Further chromatographic and spectroscopic investigation on *M. lanceolata* saponin fractions resulted in the isolation and identification of 10 new acylated triterpenoid saponins. These acylated triterpenoid saponins constituted a structurally consistent series of mono-, di- and triesters and were named **maesasaponins** (Apers et al., 1999). Further investigations using HPLC-MS lead to the characterization of an additional five triterpene saponins. However, these structures have not yet been confirmed with NMR (Fig. 1) (Theunis et al., 2007).

Maesasaponins prove to have many interesting biological activities, including virucidal activity (Sindambiwe et al., 1998; Sindambiwe et al., 1999; Apers et al., 2001), fungicidal activity (Sindambiwe et al., 1998; Okemo et al., 2003; Sisay et al., 2012), and molluscicidal activity (Sindambiwe et al., 1998; Apers et al., 2001; Bagalwa and Chifundera, 2007). However, to date mainly *in vitro* tests were performed because of the cytotoxic and haemolytic side effects of the saponins. The antiangiogenic property of maesasaponins is the most interesting biological activity in terms of pharmaceutical application (Apers et al., 2002; Foubert et al., 2012). Angiogenesis is the development of new blood vessels from pre-existing microvasculature. Besides its importance in physiological processes such as embryonic development, wound healing and menstruation, it also has a major impact in several pathophysiological conditions such as cancer, psoriasis, arthritis and retinopathy. Inhibition of angiogenesis could therefore be a novel therapeutic strategy for a number of diseases. Of all *M. lanceolata* saponins, maesasaponin II seems to be the most promising

one. It has the strongest antiangiogenic activity (Apers et al., 2002) with no haemolytic activity, not even at the highest concentration tested (20 µg/mL) (Apers et al., 2001).



Saponin	R1	R2	R3	R4
Maesasaponin I	H	angeloyl	H	H
Maesasaponin II	acetyl	angeloyl	H	H
Maesasaponin III.1				H
Maesasaponin III.2	H	angeloyl	acetyl	H
Maesasaponin IV.1				H
Maesasaponin IV.2	acetyl	angeloyl	acetyl	H
Maesasaponin IV.3	H	angeloyl	propanoyl	H
Maesasaponin V.1				H
Maesasaponin V.2	acetyl	angeloyl	propanoyl	H
Maesasaponin V.3	H	angeloyl	butanoyl	H
Maesasaponin VI.1				H
Maesasaponin VI.2	H	angeloyl	angeloyl	H
Maesasaponin VI.3	acetyl	angeloyl	butanoyl	H
Maesasaponin VII.1	acetyl	angeloyl	angeloyl	H
Maesasaponin VII.2				H
Maesabalide I	H	benzoyl	(Z)-cinnamoyl	(1-2)-α-L-rhamnose
Maesabalide II	H	angeloyl	(Z)-cinnamoyl	(1-2)-α-L-rhamnose
Maesabalide III	H	benzoyl	(Z)-cinnamoyl	(1-2)-α-L-rhamnose
Maesabalide IV	H	angeloyl	(Z)-cinnamoyl	(1-2)-α-L-rhamnose
Maesabalide V	acetyl	benzoyl	(Z)-cinnamoyl	(1-2)-α-L-rhamnose
Maesabalide VI	acetyl	angeloyl	(Z)-cinnamoyl	(1-2)-α-L-rhamnose

Fig. 1 Structures of the known maesasaponins and maesabalides. Figure adapted from Foubert et al. (2009).

In addition to maesasaponins, six related triterpenoid saponins, namely maesabalides from *M. balansae* were identified during a random drug screening program (Fig. 1) (Germonprez et al., 2004). The methanolic extract of *M. balansae* leaves have shown that these metabolites are effective in curing visceral leishmaniasis in mice and hamsters

which is comparable to that of the commercial drug against leishmaniasis, liposomal amphotericin B (Maes et al., 2004).

Concerning their biological activities, the aglycon mixture has also been tested but had very low activity compared to the normal saponin mixture. So it seems that glycosylation at position C3 is essential for the biological activities of the maesasaponins (Sindambiwe et al., 1998). Many of the observed activities are probably caused by interaction of the saponins with cell membrane components or, in the case of viruses, through interaction with viral envelope components (Sindambiwe et al., 1998).

The presence of saponins in *M. argentea* and *M. perlarius* was first identified by Foubert et al. (2009). Some of the saponins were confirmed through comparison of the molecular weight, MS fragmentation pattern and retention time with those of reference samples of maesasaponins and maesabalides. Furthermore, a butanol extract of leaves and twigs from these species showed strong anti-leishmanial action in an *in vitro* test with *L. infantum* (Foubert et al., 2009). This study provides some idea about the saponins in *M. argentea* and *M. perlarius*, though more in depth investigations are necessary to obtain conclusive data on the saponins produced in these two species.

Currently, the medicinal uses of *Maesa* are based on collecting from natural resources. It means that the supply of raw material depends on seasonal availability, species abundance, plant growth rate and other uncontrolled environmental conditions. In addition, high-value secondary metabolites are often found in low abundance in nature, thus unreliable to fulfill the needs of pharmaceutical industry (Roberts, 2007). To preserve global biodiversity and alleviate problems associated with field production, plants can be cultivated either through conventional cultivation or via plant cell and tissue cultures. Tissue cultures provide the advantage that plants are usually clones and the investigation of desirable medicinal compounds production is better amenable for standardization. Several secondary metabolites have been successfully produced on a commercial scale using plant cell culture (reviewed by Wilson and Roberts (2012)). However, the production of secondary metabolites through plant cell and tissue culture is sometimes limited by low product yield, cell culture variability and unpredictable scale-up (Chandra and Chandra, 2011).

The in vitro culture and propagation method for *Maesa* species were successfully established (Faizal et al., 2011). Using in vitro grown material, we studied the saponin production in more detail and in addition to determined saponin production in greenhouse

grown plants. Furthermore, the effect of factors possibly influencing saponin content, including organ type, age and phytohormone treatments was also investigated.

2. Results

Saponin production in *Maesa* greenhouse grown plants

Saponin content in different organs

To analyze the presence of saponins in different organs, crude extracts were made from leaves, stems and roots of 1 month old *M. argentea, M. balansae, M. lanceolata* and *M. perlarius*. These were subjected to thin layer chromatography (Fig. 2).

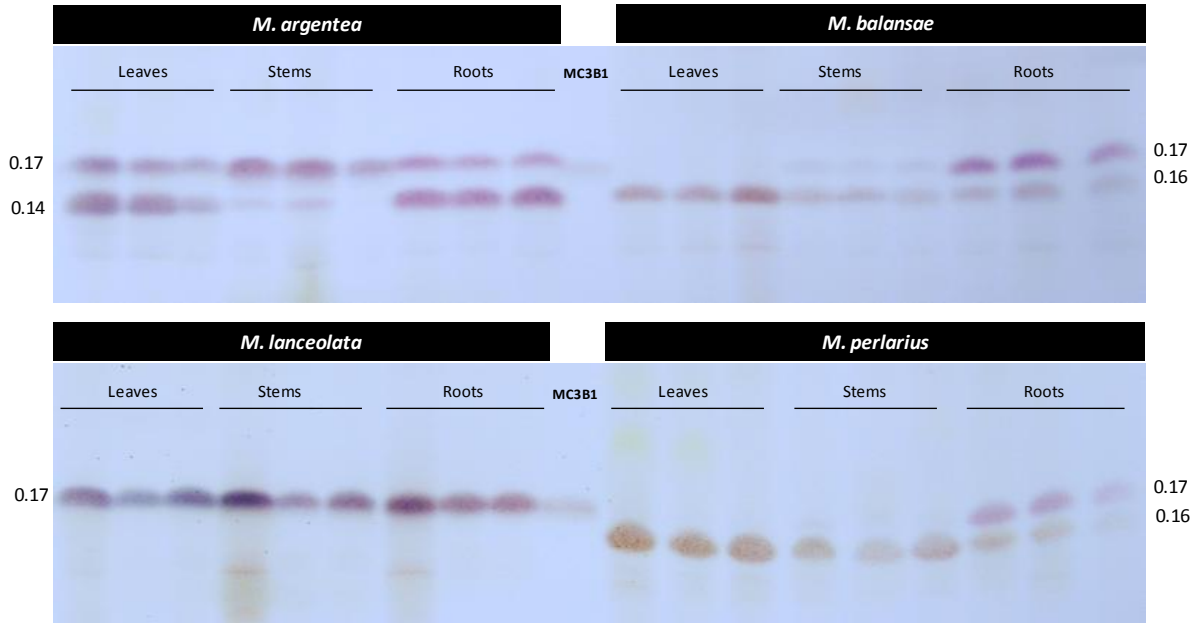


Fig. 2 TLC analysis from different organs of *M. argentea*, *M. balansae*, *M. lanceolata* and *M. perlarius*. For each species, three independent plants were used for extraction. An HPLC purified maesasaponin mix (0.1% MC3B1) was used as reference sample.

Using purified *M. lanceolata* saponin mixture (MC3B1) as a reference standard, qualitative TLC analysis showed that saponin from *M. lanceolata* was characterized by the appearance of single spot at R_f = 0.17. *M. balansae* and *M. perlarius* produced a similar profile consisting of a major spot with R_f = 0.16 in their leaves and stems and a minor spot with higher R_f values (0.17) in roots, probably the same compounds observed in the *M. lanceolata* extracts. In the previous study, liquid chromatography-mass spectrometry (LC-

MS) analysis showed that *M. perlarius* produced saponins with the same retention time and molecular weight as the maesabalides, saponin from *M. balansae* (Foubert et al., 2009). *M. argentea* showed a different profile with two major compounds with R_f = 0.14 and 0.17. The presence of a spot at R_f = 0.17 is in agreement with the LC-MS results showing that *M. argentea* leaves contained maesasaponin I, III.2, IV.3, V.3 and VI.2. (Foubert et al., 2009). On the other hand, a spot at R_f = 0.14 being the most abundant in leaves and roots suggests that *M. argentea* produces saponins quite distinct from the maesasaponins described for *M. lanceolata.*

For *M. balansae* and *M. perlarius* the colors of the two bands were slightly different. This could indicate a small difference in the aglycon structure or in attached sugar moiety. We used a normal phase TLC plate and therefore we can only conclude that the lower components are the more polar ones. This greater polarity could be caused by more sugars, other sugars or other groups on the aglycon with more oxygen atoms, for example an alcohol or acid group.

To further identify the presence of saponin in *Maesa* as has been detected with TLC, we performed HPLC-MS using extracts from 3 months old plants. The identification of the saponins was carried out by comparing the molecular weight (MW), retention time (RT), and MS^2 fragmentation pattern. MC3B1 (maesasaponins mixture from *M. lanceolata*) and PX-6518 (maesabalide mixture from *M. balansae*) served as reference samples. An overview of the saponins detected in different organs of *Maesa* is shown in Table 1. Typical HPLC profiles of *Maesa* from leaf extract are shown in Fig. 3a-d. Base peak chromatograms from *M. argentea* organs confirmed the presence of maesasaponin I, III.2, IV.3, V.3 and VI.2. In contrast to the MC3B1 sample, maesasaponin II was not detected in *M. lanceolata* organs. This could be explained by the fact that maesasaponin II was only a minor compound as reported by Theunis et al. (2007). The accumulation of this maesasaponin in young tissue probably is not sufficient to be detected in HPLC since we could detect it in older plant tissues (data not shown). Several maesasaponins in *M. lanceolata* were also detected in specific organs. For example, maesasaponin III.1, IV.2 and V.1 were not found in stems, while maesasaponin IV.2 and V.2 were not present in root extracts. Theunis et al. (2007) reported that maesasaponin IV.2 was only detected in leaves, similarly to our results. In contrast, maesasaponins IV.1 and V.1 were detected in all organs of *M. lanceolata*, whereas

a previous report showed that these maesasaponins were only detected in roots (Theunis et al., 2007).

Table 1 LC-MS results of saponins present in different organs of 3 month old *Maesa* spp.

Saponins	*m/z*	*M. argentea*			*M. balansae*			*M. lanceolata*			*M. perlarius*		
	$[M-H]^-$	L	S	R	L	S	R	L	S	R	L	S	R
Maesasaponin I	1233	X	X	X	-	-	-	X	X	X	-	-	-
Maesasaponin II	1275	-	-	-	-	-	-	-	-	-	-	-	-
Maesasaponin III.1	1305	-	-	-	-	-	-	X	-	X	-	-	-
Maesasaponin III.2	1275	X	X	X	X	X	X	X	X	X	X	X	X
Maesasaponin IV.1	1247	-	-	-	-	-	-	X	X	X	-	-	-
Maesasaponin IV.2	1317	-	-	-	X	X	X	X	-	-	-	-	-
Maesasaponin IV.3	1289	X	X	X	-	-	-	X	X	X	X	X	X
Maesasaponin V.1	1259	-	-	-	-	-	-	X	X	X	-	-	-
Maesasaponin V.2	1331	-	-	-	-	-	-	X	X	-	-	-	-
Maesasaponin V.3	1303	X	X	X	-	-	-	X	X	X	X	X	X
Maesasaponin VI.1	1337	-	-	-	-	-	-	X	-	X	X	X	X
Maesasaponin VI.2	1315	X	X	X	-	-	-	X	X	X	X	X	X
Maesasaponin VI.3	1345	-	-	-	-	-	-	X	X	X	-	-	-
Maesasaponin VII.1	1357	-	-	-	-	-	-	X	-	X	-	-	-
Maesasaponin VII.2	1313	-	-	-	-	-	-	X	X	X	-	-	-
Maesabalide I	1531	-	-	-	-	-	-	-	-	-	-	-	-
Maesabalide II	1509	-	-	-	-	-	-	-	-	-	-	-	-
Maesabalide III	1531	-	-	-	X	X	X	-	-	-	X	X	X
Maesabalide IV	1509	-	-	-	X	X	X	-	-	-	X	X	X
Maesabalide V	1573	-	-	-	X	X	-	-	-	-	X	X	X
Maesabalide VI	1551	-	-	-	X	X	X	-	-	-	X	X	X

X = presence of a molecule with identical molecular weight, retention time and MS^2 fragmentation pattern as indicated in the left column. L = leaves; S = stems; R = roots.

In *M. balansae* and *M. perlarius,* which are previously known to produce maesabalides, almost all organs showed the same type of saponins. Interestingly, investigation of the saponin in *M. balansae* and *M. perlarius* lead to the identification of several maesasaponins which have not been described for these species. Moreover, the detection of some maesasaponins also confirmed the presence of a TLC band that corresponded to MC3B1 standard. However, we could not explain why the TLC bands were only detected in roots and not in leaves and stems since the presence of maesasaponins was confirmed in all organs.

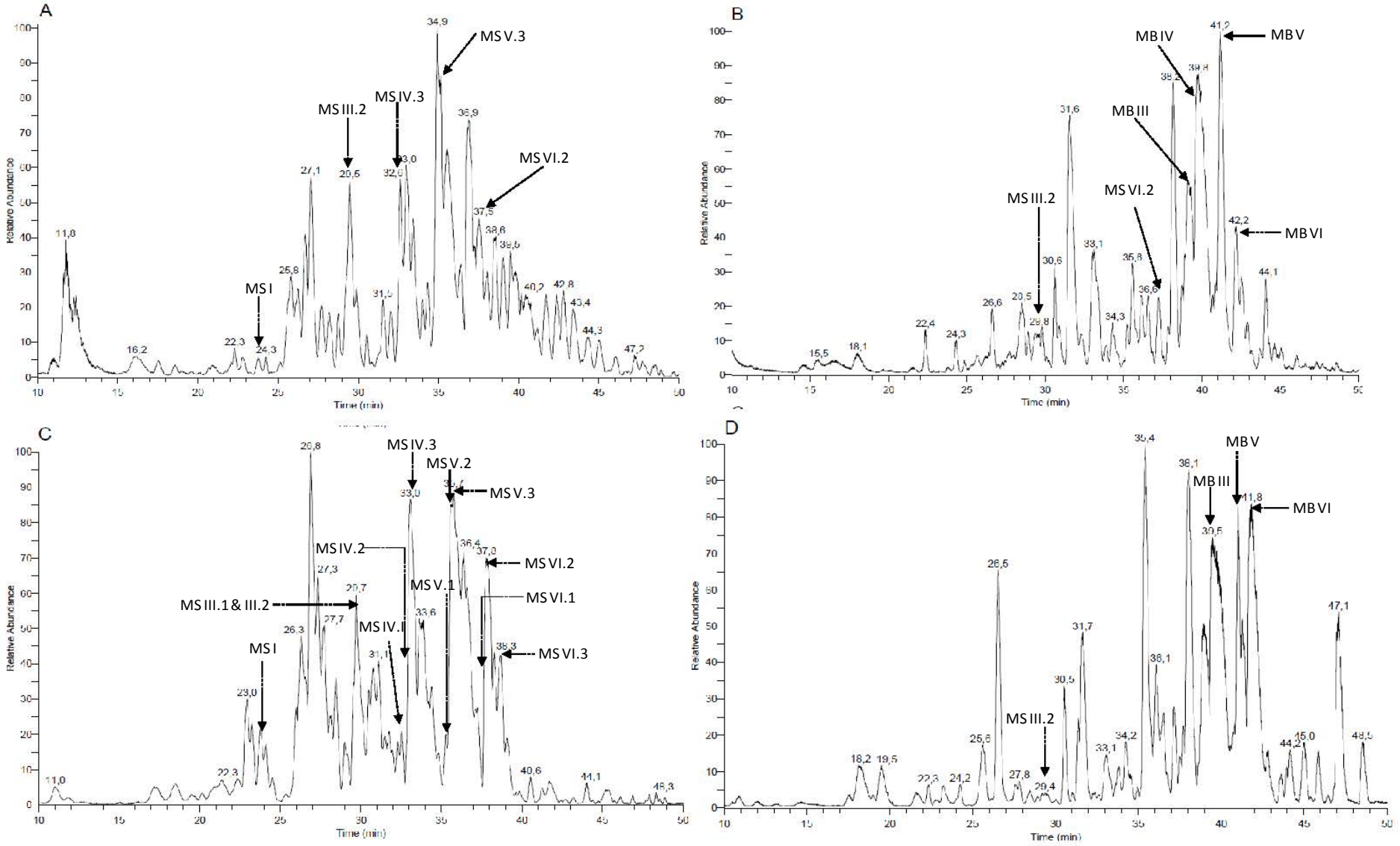


Fig. 3 LC-MS chromatogram from leaves of (a) *M. argentea*, (b) *M. balansae*, (c) *M. lanceolata* and (d) *M. perlarius*. MS = maesasaponin, MB = maesabalide.

In addition to the known saponins, the LC-MS analysis of leaf extracts from *Maesa* revealed the presence of other masses corresponding to saponins (Supplementary Table S1). Whether this is an indication of different glucosylation, hydroxylation and or acylation which might contribute to other important activities still needs to be further examined. However, based on this analysis, it is clear that all four *Maesa* species contain more differences in saponins than previously assumed.

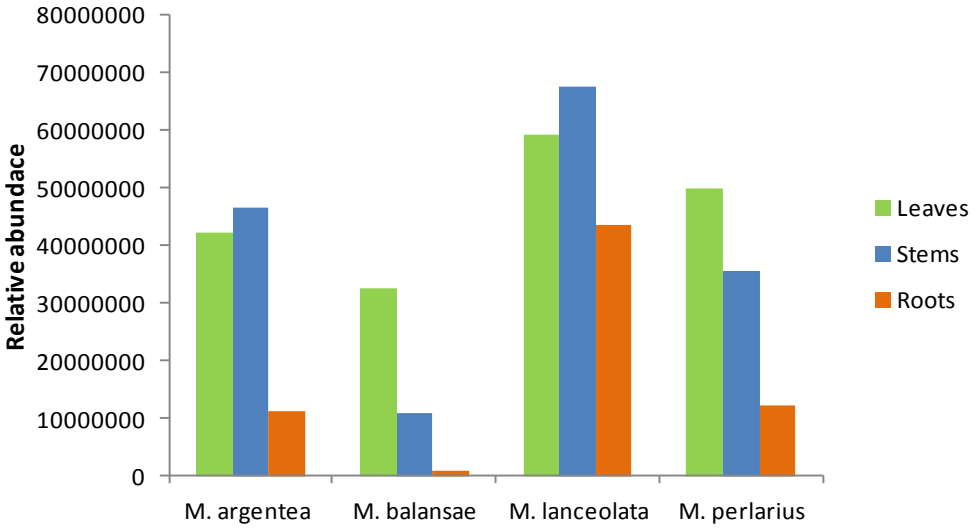


Fig. 4 Relative abundance of known saponins in different organs of *Maesa* species (n=1) calculated by the integration of the base peak chromatogram. The amounts calculated are relative to dry weight of the extracted material since we didn't have an internal standard that can allow the calculation of real concentrations.

Furthermore, to determine the relative abundance of the saponins different organ, we performed the integration of the base peak chromatogram of each individual peak corresponding to maesasaponins and maesabalides (Fig. 4). *M. argentea* accumulated saponins in stems and leaves, and less in roots. A similar pattern of accumulation was found in *M. lanceolata*. Both *M. balansae* and M. *perlarius* predominantly accumulated saponins in leaves followed by stems and roots. In a previous report leaves and roots of greenhouse grown *M. lanceolata* plants have also been analyzed for their saponin content (Theunis et al., 2007). The amount of total saponins in young leaves of greenhouse grown plants (4.9% ± 0.8; $n = 3$) was much higher than the amount in the roots of the same plants (1.49% ± 0.25). Although, by comparing the saponin extracts of plants grown in our greenhouse using TLC with a maesasaponin standard (MC3B1), we found that our plants

probably did not contain similar high amounts of saponins. Furthermore, we could not draw a conclusion on the total content of saponin from each organ since the sample size of each experiment was not statistically adequate (n = 1).

Saponin content in Maesa plants with different ages

Maesa species tested in the previous experiments are from 3 month old plants. However, seedlings or juvenile plants can be physiologically quite different from adult plants. To investigate whether saponin content varies with age, we analyzed saponin content from leaves of greenhouse grown plants from approximately 1 month, 9 months and 2 years old. TLC results are shown in Fig. 5.

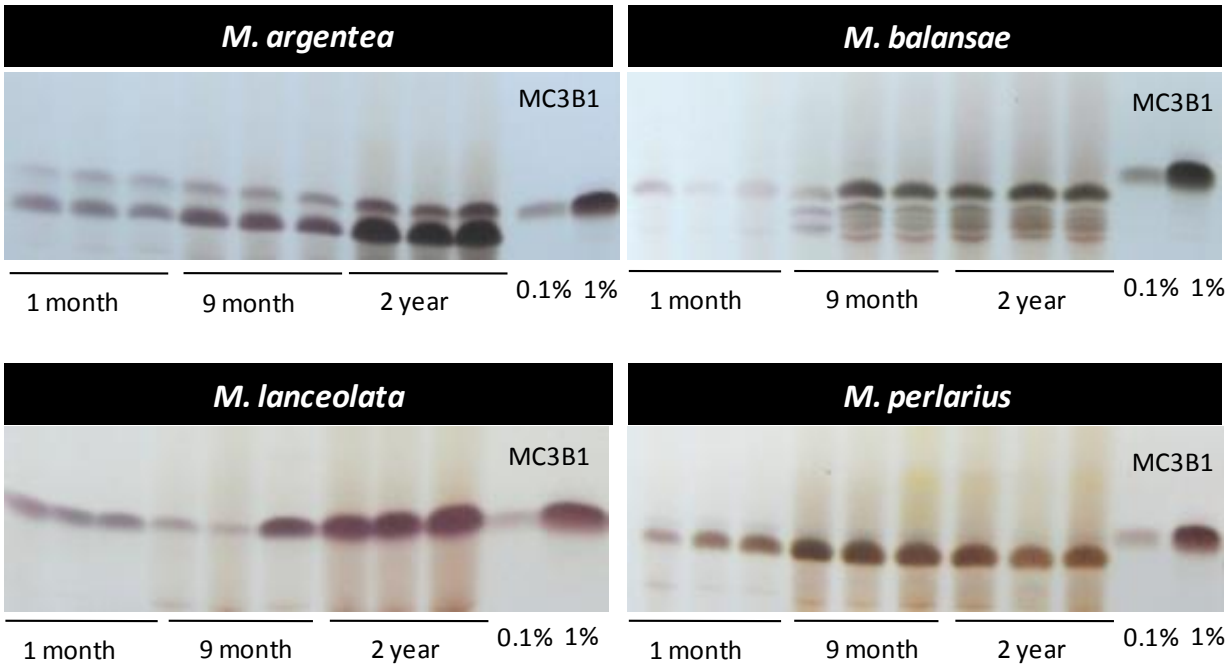


Fig. 5 TLC analysis of saponin extracts from leaves of *M. argentea*, *M. balansae*, *M. lanceolata* and *M. perlarius* plants growing in the greenhouse for 1 month, 9 months and 2 years. An HPLC purified maesasaponin mix (MC3B1) was used as reference sample. The three repeats correspond to extracts from individual plants.

For *M. argentea* two characteristic bands, with R_f 0.14 and 0.17, were obtained. For both bands, the intensity and surface area increased as the age of the plants increased. For *M. balansae*, the spot with R_f 0.16 was more pronounced in leaves of 9 month and 2 year old plants, compared to 1 month old plants. Besides the band with R_f 0.16, smaller bands with lower retention factors were also present. *M. lanceolata* had one band with R_f 0.17

and, although there was some variation between different plants of the same age, it was clear that leaves of 2 year old plants had a higher saponin content than leaves of the younger plants. For *M. perlarius* one large spot with R_f 0.16 was detected that was larger and more intense in 9 month and 2 year old plants. In general, we conclude that saponin production in leaves of *Maesa* plants markedly increased with increasing age of the plants.

Saponin production in in vitro cultures

Saponin production in regenerated shoots

Because we were interested in the saponins produced by *Maesa* species, it was important to assess the capacity to produce saponin in in vitro cultivated plants. To investigate the saponin content, extracts from leaves of regenerated plantlets of all four *Maesa* species and of the corresponding in vitro control plants were compared using TLC (Fig. 6). A qualitative TLC analysis showed that regenerated shoots produced a phytochemical profile similar to shoots of greenhouse plants.

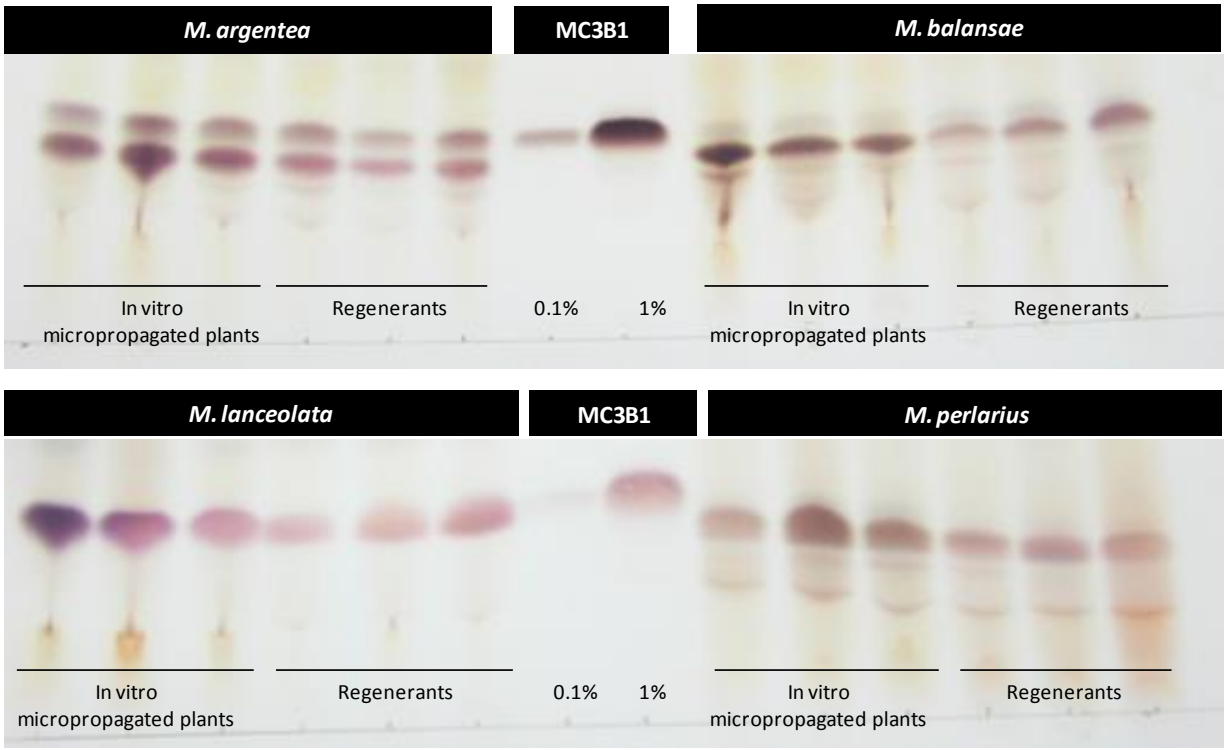


Fig. 6 TLC analysis of saponin production in in vitro micropropagated plants and regenerants induced through adventitious shoots induction for *M. argentea*, *M. balansae*, *M. lanceolata* and *M. perlarius*. MC3B1 is an HPLC purified saponin mixture of *M. lanceolata* saponins and is used here as a reference sample 0.1% and 1% (w/v). Different repeats represent different individual plants.

Saponin content was also determined for other types of in vitro cultures, namely hairy roots and using TLC. In hairy roots the signal was much weaker suggesting lower saponin content in hairy roots compared to shoots (Lambert, 2011). In callus extracts, however, no saponin signal was detected (Chapter 7).

The influence of phytohormones on in vitro culture

Phytohormones play important roles in regulating developmental processes and signaling networks involved in plant responses to a wide range of biotic and abiotic stresses. They are also very often used in tissue culture techniques. Therefore, plant hormones are a potential source of variation in terms of secondary metabolite accumulation in in vitro plant cultures. Several reports have described the upregulation of secondary metabolites upon treatment with phytohormones (Zhao et al., 2005; Kim et al., 2007; Liu et al., 2007; Coste et al., 2011). Therefore, it would be interesting to see if plant hormones such as gibberellic acid (GA3), 2,4-dichlorophenoxyacetic acid (2,4-D), and abscisic acid (ABA) can influence saponin biosynthesis in *Maesa*.

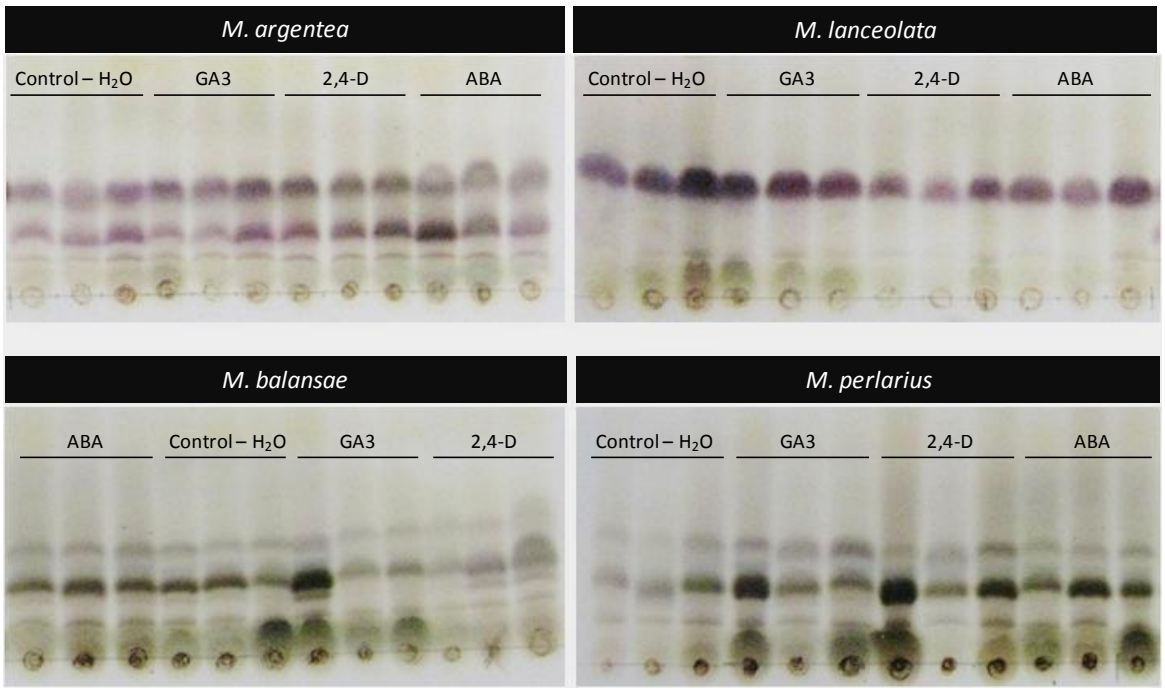


Fig. 7 TLC of in vitro plantlets of *M. argentea*, *M. balansae*, *M. lanceolata* and *M. perlarius* upon exposure with different hormones; 0.01 mM gibberellic acid (GA3), 0.01 mM 2,4-dichlorofenoxyacetic acid (2,4-D) and 0.01 mM abscisic acid (ABA). Controls were treated with water and all samples were harvested 48 h after treatment. Different repeats represent separate plants.

None of the plants showed major morphological changes after treatment with any of the phytohormones within the time of analysis (Fig. 7). Therefore, we assume that short hormone treatments do not strongly influence metabolite sinks. Treatment with GA3, 2,4-D and ABA did not lead to major changes in saponin content as determined by TLC. Although there were differences between the controls and the treated samples, these were most probably due to the extraction and/or TLC procedure. To address the problem of inadvertent technical mistakes, each treatment was analyzed by extracting saponins from three individual plants separately. The variations occurring in samples from treated plants were also noticed in control extracts.

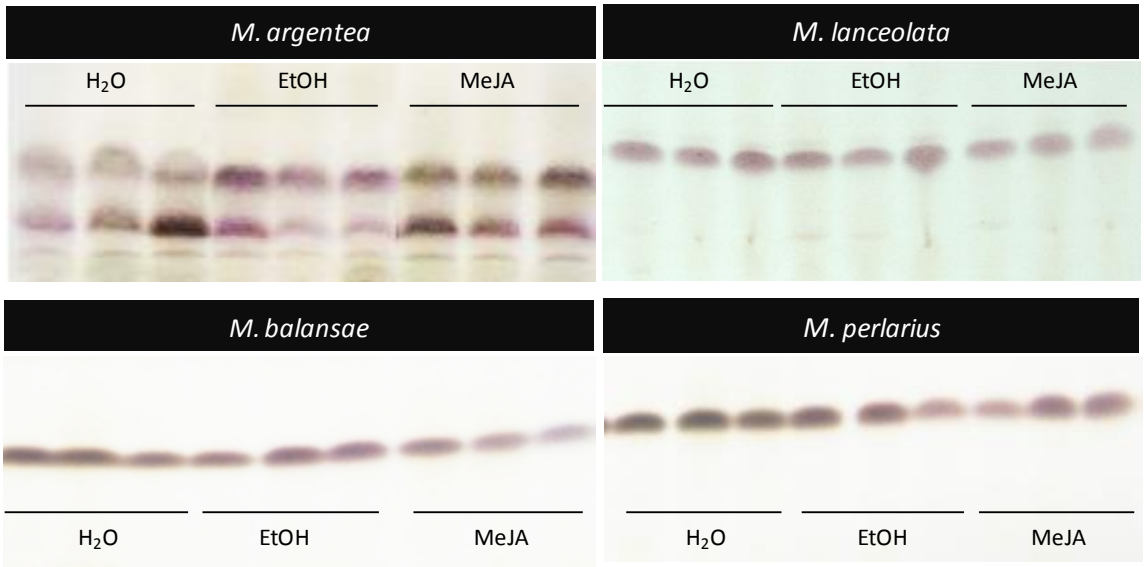


Fig. 8 TLC of in vitro plantlets of after exposure with 0.1 mM methyl jasmonate (MeJA). Control leaves were treated with H_2O and with ethanol. Samples were harvested 48 h after treatment. Different repeats represent different plants

In addition to the phytohormones, we also tested other hormones, which are widely used for elicitation of plant secondary metabolite: methyl jasmonate (MeJA) and salisylic acid (SA). Jasmonates are phytohormones derived from the metabolism of membrane fatty acids, which are widespread in the plant kingdom. These compounds are involved in crucial processes related to plant development and survival, including, senescence, reproduction, fruit development, direct and indirect defense responses, and secondary metabolism (Wasternack, 2007; Song et al., 2011; Woldemariam et al., 2011). Among jasmonates, jasmonic acid (JA) and methyl jasmonate (MeJA) are commonly used for enhancing secondary metabolites production like saponins (Hayashi et al., 2003; Kim et al., 2004; Ali et

al., 2006; Shabani et al., 2009; Bonfill et al., 2011). Therefore, we tested the effect of 0.1 mM MeJA on in vitro shoots of all four *Maesa* species and investigated saponin production with TLC (Fig 8). Plantlets were dipped in a solution for 30 sec and then placed on fresh medium.

TLC analyses showed that there were no increases in saponin concentration of four *Maesa* species. These results are in contrast with MeJA effects on other secondary metabolites which often increase upon MeJA treatment (Suzuki et al., 2005; Mangas et al., 2006; Satdive et al., 2007; Shabani et al., 2009). This demonstrated that *Maesa* saponins are produced in a stable fashion and not affected by MeJA treatment.

Salicylic acid is another important phytohormone that plays a role in various physiological responses in plants (Vicente and Plasencia, 2011) and in response to biotic stresses and pathogenesis (Vlot et al., 2009). Furthermore, SA also participates in the signaling of abiotic stress responses, such as drought, high and low temperature, salinity, ozone, UV radiation, and heavy metals (Hara et al., 2012). Because of these roles, SA is often used for the elicitation of plant secondary metabolites. For this, *Maesa* in vitro plants were submerged in an aseptic SA solution for 30 sec, samples were harvested 48 h after treatment and saponin production was examined with TLC (Fig. 9).

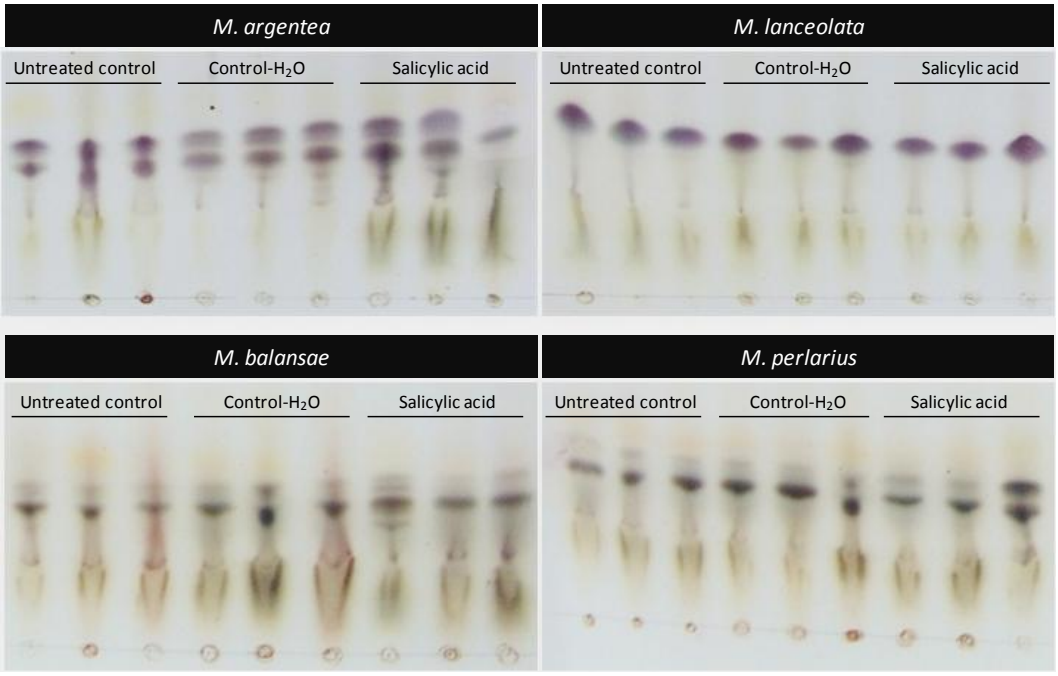


Fig. 9 TLC of in vitro plantlets of *M. argentea* , *M. balansae* , *M. lanceolata* and *M. perlarius* exposed with 0.01 mM salicylic acid for 48 h. As a control, non-treated leaves and leaves treated with water were used. Different repeats represent different plants.

Qualitative TLC analyses indicated that there were no changes in saponin content observed in all four *Maesa* species after treatment with SA. The results are in agreement with a stable accumulation of saponins in *Maesa* plants, not altered by externally applied phytohormones.

3. Discussion

TLC and LC-MS analyses of *Maesa* greenhouse grown plants revealed that saponins were present in leaves, stems and roots of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius*, although, there were considerable qualitative and quantitative variations observed between different organs. These differences between organs have also been reported in the literature for *M. lanceolata* and also for other species such as *Medicago trucantula* and *Avena* spp. (oats). For example, saponin accumulation in the leaves of *M. lanceolata* is 3 fold higher than in the roots (Theunis et al., 2007). Similarly, conjugates of the triterpene saponin medicagenic acid were highly accumulated in leaves of *M. trucantula*, while soyasapogenol was higher in the root (Huhman et al., 2005). In oats, the distribution of the two groups of saponins produced is mutually exclusive since avenacosides (steroidal saponins) are produced in the leaves and avenacins (triterpenoid saponins) in the roots (Osbourn, 2003). The differential distribution of specific saponins is most likely the result of spatially controlled biosynthesis, specific function, and active transport.

In addition to differences in saponin content in different organs, we also observed differences in saponin concentration depending on the developmental stage. Older plants produced more saponins compared with younger plants. This confirms that plant age is one of the important factors influencing saponin content as has been reported in many plant species. In *Medicago sativa*, the concentration of medicagenic acid and zanhic acid respectively increased by nearly 1.5 and 2 fold passing from the second to the third year of growth (Pecetti et al., 2006). The age of harvested plants is also essential for all species of ginseng. The amount of ginsenosides increased from 1 to 5 year old roots of *P. ginseng* (Shi et al., 2007) and *P. quinquefolius* (Qu et al., 2009). Despite this, the age of the plants and saponin content is not always positively correlated. For example, the content of saponins in *Polygala tenuifolia* decreased with increasing root age and therefore, annual roots had the

highest quality (Teng et al., 2009). Likewise, 1 year old roots of *Bupleurum chinense* had a higher quality than that of 2 year old roots due to their higher saikosaponin content (Tan et al., 2008).

Despite saponins being widely distributed in plants, so far, the production of saponin using tissue culture cultivation has been limited to ginseng. Zhong et al. (2000) reported that the triterpenoid saponin ginsenosides content in cell suspension cultures of *Panax notoginseng* in a bioreactor is between 20 and 45 mg/g dry weight. This was comparable to roots from in vivo grown plants of *P. ginseng* that contain 20 to 56 mg/g dry weight of the ginsenosides (Wu and Zhong, 1999).

To bridge this gap, we have established different types and conditions for in vitro culture of four *Maesa* species (Faizal et al., 2011; Lambert, 2011). We have also analyzed the saponin content in hairy roots and undifferentiated callus using TLC. In hairy roots the signal was much weaker suggesting lower saponin content in hairy roots compared to shoots, while no saponins were presence in callus extracts, (Lambert, 2011). Although the saponin detection method applied here does not allow a solid assessment of the absolute amount of saponin produced, the TLC results provide strong indications that saponin production is low in hairy roots and callus and that *in vitro* regenerated shoots maintained a significant capacity to produce and accumulate saponins. Regenerated shoots that were acclimatized and grown in the greenhouse for several weeks accumulated saponins at a concentration similar to what was detected in plants that were cultivated over long periods (data not shown). Together, the results show that in vitro grown shoots are similar to in vivo plants with regard to morphological characteristics and saponin biosynthesis.

In addition to being the most studied species for in vitro analysis of saponins, *P. ginseng* has also been studied for phytohormone enhancement of saponin production. Treatment of *P. ginseng* adventitious root with 0.025 mM indole-3-butyric acid (IBA) increased saponin content 1.6 fold (Kim et al., 2007). In *P. quinquefolium* and *P. japonicus* cell cultures, the content of saponins varied depending on the hormones in the culture medium (Zhong et al., 1996; Smirnova et al., 2010). However, short treatment of *Maesa* in vitro shoots with three different types of hormones; GA3, ABA and the auxin 2,4-D had no effect on saponin production. On the other hand, we observed that long term treatment with 2,4-D alone or in combination with cytokinins resulted in subsequent callus formation,

for which we showed that they did not produce saponins (chapter 7). Though, how the saponin content is reduced or degraded in callus need to be further investigated.

We have also shown that saponin content was higher in more mature leaves and older plants. Therefore, it may be suggested that auxin and/or gibberellic acid are responsible for the increase in saponin production since these growth regulators are generally involved in leaf growth. However, a short contact of in vitro *Maesa* leaves with auxin or gibberellic acid did not significantly change saponin production. Consequently, the increased saponin production in mature *Maesa* leaves cannot be a direct effect of the higher auxin and/or gibberellic acid levels in leaves.

Another important method to enhance saponin production is via treatment with elicitors (reviewed by Yendo et al. (2010) and Lambert et al. (2011)). The elicitation process is generally regarded as the expression of defense-related genes and activating defense-related secondary metabolic pathways (Pauwels et al., 2009). Elicitation is a very complex process and depends on many factors such as elicitor concentration, growth stage of the culture at the time of elicitor addition and contact time with the elicitor (Vasconsuelo and Boland, 2007; Sivanandhan et al., 2012). In addition, the response to a particular elicitor may vary from plant to plant (Vasconsuelo and Boland, 2007). In this context, we studied the effect of MeJA and SA as elicitors on saponin accumulation from four *Maesa* species beside their roles as phytohormones. The addition of JA and/or MeJA has been reported to strongly enhance production of ginsenoside, saponin in adventitious root cultures of *P. ginseng* (Yu et al., 2002) and the accumulation of saponin in whole plant culture of *Centella asiatica* (Kim et al., 2004).

Regarding an SA elicitation, its beneficial effect has been reported in many plant species. Treatment of root biomass with 150 µM SA for 4 h exposure resulted in significantly improved withanolides production in the adventitious root cultures of *Withania somnifera* by 10 to 50 fold (Sivanandhan et al., 2012). The addition of 20 µM SA to *Saussurea medusa* cell cultures resulted in flavonoid production about 2.5 fold higher than the control (Yu et al., 2006). Likewise, Pu et al. (2009) reported that treatment of SA from 24 to 96 h also stimulated *Artemisia annua* growth and artemisinin production. In contrast, none of these treatments show significant increase in saponin production from *Maesa*.

In addition to the previous mentioned elicitors, we have also performed a screen of many different putative elicitors, which may have had potential to enhance saponin

production in *M. lanceolata* hairy roots (Lambert, 2011). However, HPLC-MS revealed that none of the substances induced saponin production. Therefore, we conclude that *Maesa* saponins are constitutively present in plants. The level of these compounds is significantly influenced by intrinsic factors reflecting the physiological status of the plants, in particular the developmental stage and age of the plant. In addition, they are produced in a stable manner irrespective of external factors or environmental stimuli.

4. Material and Methods

Plant material

M. lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). The in vivo plants were maintained in the greenhouse of Department of Plant Production, Ghent University. The in vitro plants were maintained as previously described in Chapter 2.

Hormone treatment of in vitro shoots

ABA, 2,4-D, GA3 and SA were tested at 0.01 mM and were dissolved in water. MeJA was dissolved in 100 % ethanol and used at 0.1 mM.

Plantlets were removed from the culture medium in the tissue culture containers and were submerged in the phytohormones or control solutions for 30 seconds. Afterwards, every plant was placed into a glass tube containing solid MS medium supplemented with MS vitamins, 3% (w/v) sucrose and 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham, UK). The cultures were placed in a growth room with 16/8 h light/dark conditions at 26 °C. Samples (1^{st} – 6^{th} leaves, starting from the apex) were harvested for saponin extraction 48 h after treatment. Saponin content was investigated with TLC.

Saponin analysis

Thin layer chromatography

For TLC analysis, 50 mg dry weight of plant material was ground with liquid nitrogen. 250 µL of 50% (v/v) methanol was added and samples were sonicated for 1 h. Subsequently, extracts were centrifuged at 13000 rpm for 10 min and the supernatant was transferred to a fresh eppendorf tube. The pellet was resuspended with 250 µL 50% (v/v) methanol and

sonicated for another hour. Samples were centrifuged at 13000 rpm for 10 min and the supernatant was combined with the supernatant from the first step. Samples were dried for 3 – 4 h using a vacuum concentrator (Heto VR-I, High Technology of Scandinavia) attached to a Savant RT4104 refrigerated condensation trap. Afterwards, the pellet was resuspended in 50 µL 80% (v/v) methanol. This extract was further used for TLC analysis.

TLC analysis was performed with normal phase silica gel 60 plates with fluorescence indicator (F_{254}) (Merck KGaA, Germany). For the mobile phase, the upper layer of a mixed of n-butanol/acetic acid/H_2O (40/10/50) was used. One hour before starting the TLC run, the mobile phase was brought into the TLC tank and a filter paper was placed at the back of the tank. 10 µL of the samples was spotted at 2 cm from the sides and bottom of the plate. Afterwards, the plate was placed in the tank for 4 h. The saponins were stained using an anisaldehyde reagent (5 mL/L p-anisaldehyde, 100 mL/L acetic acid, 850 mL/L methanol and 50 mL/L sulphuric acid). The reagent was sprayed onto the TLC plate using an EcoSpray (Carl Roth GmbH). Saponin spots were visible 10 min after heating the plate to 100 °C on a hot plate.

LC-MS analysis

LC-MS analysis was performed in the Lab of Pharmacognosy and Pharmaceutical Analysis (University of Antwerp). Methanol and acetonitrile were of HPLC-grade and purchased from Acros Organics (Geel, Belgium), as was acetic acid (99.8%). Deionized water for HPLC analysis was prepared with a Millipore water purification system (Millipore, Bedford, MA, USA). A semi-pure extract of *M. balansae* (PX-6518 containing Maesabalides I, II, III, IV, V and VI) and reference compounds of *M. lanceolata* (MC3B1) containing maesasaponins were available from previous investigations (Apers et al., 1999; Germonprez et al., 2004).

Oven-dried material (leaves, stems, and roots) from 3 month old plants grown in the greenhouse were ground and extracted with MeOH 50% (v/v) by sonication, after which the extracts were dried under reduced pressure. HPLC analysis of methanol 80% solutions of the *Maesa* extracts (10 mg/mL) and the semi-pure extract of MC3B1 and PX-6518 (5 mg/mL) was performed according to the method of Theunis et al. (2007) using a Surveyor LC system equipped with a diode array detector (Thermo Fisher, San Jose, CA) and a silica-based 300 monomeric C18 column (Grace Vydac, Hesperia, USA) (250 x 3.2 mm, 5 µm). The flow rate was 0.5 mL/min, UV detection was carried out at 210 nm and the gradient program was:

solvent A: 0.05% CH_3COOH; solvent B: CH_3CN + 0.05% CH_3COOH; gradient: 10 min 25% B – from 25 to 60% B in 30 min – from 60 to 90% B in 7 min – from 90 to 25% B in 3 min – 5 min 25% B. The injection volume was 20 µL. The LC system was coupled to an LXQ linear ion trap (Thermo Fisher). The experimental conditions for operation of the instrument in the (–)ESI mode were optimized by direct infusion of a solution of maesasaponins (1 µg/mL). The optimal conditions were: sheath gas flow, 65 arbitrary units; auxiliary gas flow, 14 arbitrary units; sweep gas flow, 3 arbitrary unit; spray voltage, + 4.0 kV; ion transfer tube temperature, 350 °C; and capillary voltage, –3 V. Mass spectral data were recorded using full scanning mass range *m/z* 400-1800 and data dependent scanning with a parent mass list containing the masses of the maesabalides. For MS^n experiments an isolation width of 2 Da was used and 35% normalized collision energy was applied. All data were recorded and processed using Xcalibur software, version 2.0. (Thermo Fisher).

Acknowledgement

We thank the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia and the BOF (special research fund No. 01WI0111, Ghent University) for A.F. PhD fellowship. We also acknowledge the help from Nathalie Van Den Bleeken for improving the saponin detection protocol with TLC.

4 | Combinatorial biosynthesis of triterpene saponin in *Maesa lanceolata*

Cover art: *Maesa lanceolata* hairy root

4

Combinatorial biosynthesis of triterpene saponin in *Maesa lanceolata*

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Sandra Apers, Alain Goossens, and Danny Geelen

Authors' contributions:

[1] Generating *Maesa* hairy roots, RT-PCR, saponin extraction, analysis of data and writing the chapter.
[2] LC-ESI-FT-ICRMS and metabolite profiling.
[3] HPLC-MS analysis.

Abstract

Recent research effort is being allocated to the elucidation of the saponin biosynthesis pathway. The identification of genes and their functional analysis have provided the information required to begin with the modulation of saponin production in a more selective and direct way. For this study, we have generated hairy root lines from *Maesa lanceolata*, a tropical plant species with pharmaceutically interesting triterpene saponins. The saponin biosynthetic pathway of this species remains unidentified because little is known about genetic sequences from this plant species. Therefore, a semi-rational approach for changing the saponin production was developed. With Combiplan or combinatorial biosynthesis in plants platform, several candidate saponin biosynthesis genes from different plant species were heterologously expressed in *M. lanceolata* using overexpression cassettes. By introducing these genes, it is awaited that novel compounds are synthesized that are not normally produced in the host.

1. Introduction

For centuries, people have used plants or plant-derived products with varying success to treat and cure diseases and injuries (Raskin et al., 2002). However, only a small fraction of the immense diversity of plant metabolites has been explored for the production of new medicines and other products important to human well-being. The availability of inexpensive high-throughput sequencing is rapidly expanding the number of species that can be investigated for the speedy discovery of previously unknown enzymes and pathways (Lam, 2007). Exploitation of these resources is being carried out through interdisciplinary synthetic and chemical biology to engineer pathways in plant systems for improving the production of existing medicines and to create libraries of biologically active products that can be screened for new drug applications (Newman and Cragg, 2012).

Hairy roots

Genetically transformed hairy root cultures are more preferable over other cell or tissue culture types mostly because of their genetic and biochemical stability, and similar biosynthetic products compared to the parent plant (Georgiev et al., 2007; Chandra and Chandra, 2011). Furthermore, hairy root cultures often accumulate higher levels of phytochemicals than cell/callus cultures containing undifferentiated cells (Ono and Tian, 2011).

Hairy root is a disease in plants that are wounded and infected by the gram-negative soil bacterium *Agrobacterium rhizogenes*. When susceptible plants are wounded, they produce phenolic substances, such as acetosyringone. This substance induces the virulence (*vir*) genes in the bacterium, which are responsible for transferring the T-DNA fragments of Ri (root inducing) plasmid of *A. rhizogenes* to the plant cells. This chromosomal integration into the nuclear genome of the host plant causes neoplastic root hair proliferation (Giri and Narasu, 2000; Georgiev et al., 2007). To date, *A. rhizogenes* has been successfully used for hairy root transformation of more than 400 plant species, and this number is continuously increasing (Ono and Tian, 2011).

Normally, root cultures need an exogenous phytohormone supply and grow very slowly, resulting in the poor biomass production and hence negligible yield of secondary metabolites. However, the hairy root system is highly productive under hormone-free

culture conditions. In addition, hairy root cultures grow fast with a short doubling time and are easy to maintain. The ability to synthesize a range of chemical compounds of hairy root cultures offer additional advantages as continuous sources for the production of valuable secondary metabolites. Some of the compounds produced via hairy roots include pharmaceuticals, cosmetics, and food additives (Giri and Narasu, 2000; Guillon et al., 2006; Georgiev et al., 2007; Banerjee et al., 2012). Examples of compounds produced via hairy roots are scopolamine from *Atropa belladonna* (Bonhomme et al., 2000), artemisinin from *Artemisia annua* (Weathers et al., 2005), and antimicrobial-polyacetylenes from *Panax ginseng* (Fukuyama et al., 2012).

Combiplan: Combinatorial biosynthesis in plant

Current research in plant metabolic engineering indicates that combining the genes from different organisms could be important to generate valuable compounds. The approach to achieve this is referred to as combinatorial biosynthesis and is an innovative strategy for the generation of novel secondary metabolites, as well for the production of rare natural products (Oksman-Caldentey and Inze, 2004; Floss, 2006; Julsing et al., 2006; Menzella and Reeves, 2007; Zhang and Tang, 2008; Pollier et al., 2011). Combinatorial biosynthesis is not limited to the introduction of a single gene but can also include a series of genes with the aim to reconstruct a complete biosynthesis pathway that can impinge on an existing biosynthetic pathway in the host.

The introduction of building blocks can be achieved by recruiting different biosynthesis steps for the assembly of engineered pathways in the host system of choice, using new enzyme-substrate combinations. A combinatorial biosynthesis approach in microorganisms has previously proven successful to generate novel antibiotics. Such a strategy has not yet been implemented into a higher plant system because of the poor understanding of secondary metabolism and because of practical hurdles such as a slow life cycle compared to microorganisms. At the moment, combinatorial biosynthesis of plant secondary metabolites focuses on the reconstruction of the basic pathways into microbial hosts. Recent achievements with the plant polyketide biosynthesis by microorganisms, especially in *Escherichia coli*, demonstrated the utility of combinatorial biosynthesis (Horinouchi, 2009).

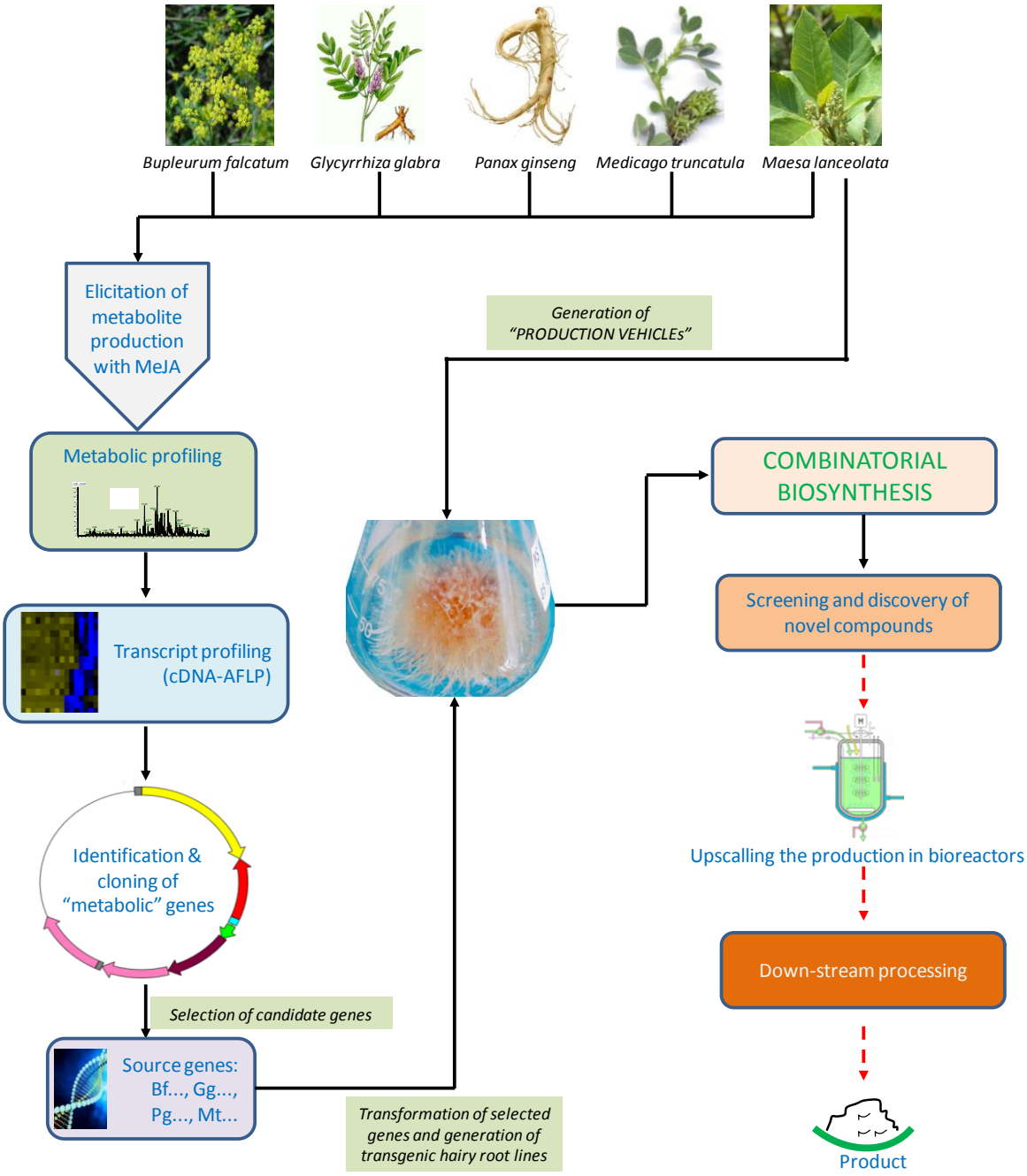


Fig. 1 A combinatorial biosynthesis approach in *M. lanceolata*.

The key objective of the Combiplan project was 'to establish a combinatorial biosynthesis platform in plants that will allow the semi-rational combinatorial engineering of the biosynthesis of existing and novel secondary metabolites in plant cell tissue cultures'. To obtain proof of concept, the combinatorial biosynthesis platform (Fig. 1) was applied to the metabolite class of triterpene saponins. Five plant species were selected based on their saponin content: *Medicago truncatula, Panax ginseng, Maesa lanceolata, Bupleurum*

falcatum and *Glycyrrhiza glabra*; with *M. truncatula* and *M. lanceolata* being the main plant species used. These species produce structurally related triterpene saponins but with varied structural features and different biological activities. Notably, suitable cell, tissue or organ cultures were generated as 'production vehicles'. Elicitors are added to these cultures for a higher production of specific secondary metabolites, which can be detected through established metabolite profiling techniques. The rationale of the project was that elicitor treatment not only enhances the production of the desired secondary metabolites, but also activates the genes involved in the biosynthesis of such compounds. cDNA-AFLP based transcript profiling identified many candidate genes involved in the production of secondary metabolites. In parallel, high-throughput transformations were developed. Subsequently, a collection of overexpression and RNAi cassettes were created for the construction of a combinatorial biosynthesis library. Finally a screening procedure was set up to find novel metabolites and novel activities.

2. Results

M. lanceolata hairy root induction

M. lanceolata hairy roots were induced using *A. rhizogenes* (strain LBA 9402/12) transformation on leaf discs. The *Agrobacterium* strain was transformed with the pK7WG2D plasmid, in which an eGFP-ER gene was inserted after a 35S promoter sequence. Hairy roots appeared from wounded sites 15-30 days after inoculation with *A. rhizogenes* (Fig. 2a-b). Uninfected control explants did not form adventitious roots. GFP was used as a visible marker to rapidly select transformed hairy roots (Fig. 2c-d). Isolated hairy roots, in contrast to untransformed roots, grew autonomously in hormone free medium.

Establishment of overexpression lines

A gene discovery program was initiated on in vitro shoots of *M. lanceolata*. Since no gene sequence resources were available for this plant, the effect of the methyl jasmonate (MeJA) treatment was initially investigated with a pilot cDNA-AFLP analysis, which confirmed a typical MeJA transcriptional reprogramming response. Next, a gene discovery program was performed on the MeJA treated in vitro shoot cultures.

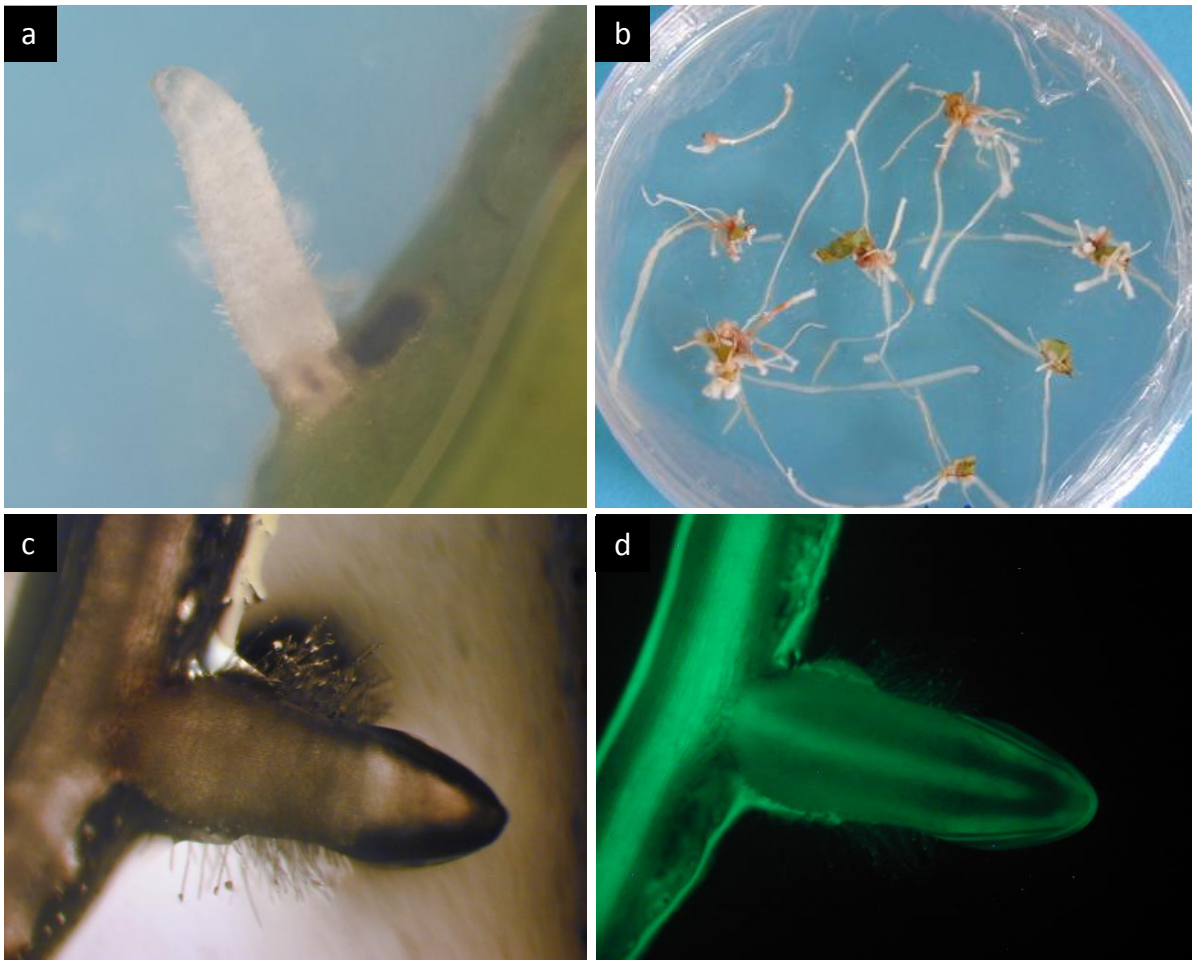


Fig. 2 *M. lanceolata* hairy roots appearing at the wounded sites 2 to 4 weeks after infection (**a** – **b**); transgenic roots were selected based on GFP fluorescence (**c** – **d**).

After BLAST analysis, a list of 73 MeJA responsive gene tags from 5 different plant species was selected as candidate genes. The group of genes consisted of oxidases, reductases, cytochrome P450s, glucosyltransferases, esterases, acyltransferases, transcription factors and some early pathway genes involved in saponin biosynthesis or its regulation. Subsequently, the full length-open reading frames (FL-ORFs) of these candidate genes were cloned into an overexpression vector (pK7WG2D) and used for transformation of *M. lanceolata* leaf discs with *A. rhizogenes*. This supporting work was executed in the Secondary Metabolites Group, Department of Plants Systems Biology-University of Ghent/VIB (PSB-UG/VIB). More technical details are shown in the Materials and methods section. An overview of the genes that were transformed in *M. lanceolata* is presented in Table 1.

Table 1 An overview of the selected cDNA-AFLP tags from *Bupleurum falcatum* (Bf), *Glycyrrhiza glabra* (Gg), *Maesa lanceolata* (Ml), *Medicago truncatula* (Mt) and *Panax ginseng* (Pg) overexpressed in *M. lanceolata*.

Code	(putative) annotation
BF0137	Cytosolic aldehyde dehydrogenase RF2C
BF0232	NADH:cytochrome b5 reductase
BF0567	CYP716A12: Cytochrome P450 monooxygenase
BF0616	4,5-DOPA dioxygenase extradiol-like protein, putative
BF0838	Short-chain dehydrogenase/reductase SDR
BF0949	Leucoanthocyanidin dioxygenase-like protein
BF1507	Hypothetical protein (IPR001087 Lipolytic enzyme, G-D-S-L protein domain)
BF1557	O-methyltransferase, family 2
GG0018	Putative leucoanthocyanidin dioxygenase
GG0043	Putative UDP-glucosyltransferase
GG0048	Putative pyridine nucleotide-disulphide oxidoreductase
GG0057	Putative polyphenol oxidase
GG0089	Putative cytochrome P450 (IPR001128 Cytochrome_P450 ;IPR002401 EP450I protein domains)
GG0142	Putative aldo/keto reductase
GG0167	Putative cytochrome P450
GG0242	Putative anthocyanin acyltransferase
GG0244	Btvl-domain containing protein
GG0347	Putative aspartate-semialdehyde dehydrogenase
GG0411	Btvl-domain containing protein
GG0426	Putative mannitol dehydrogenase 1
GG0436	Putative cytochrome P450
GG0452	Putative cytochrome P450
GG0514	Putative methylsalicylate esterase
GG0526	Putative UDP-glucosyltransferase
GG0551	Putative epoxide hydrolase
GG0628	Putative UDP-glucosyltransferase
GG0739	Putative short-chain dehydrogenase/reductase
GG0791	Putative NAD-dependent aldehyde dehydrogenase
GG0818	Putative cytochrome P450
GG0888	Putative UDP-glucosyltransferase
GG0918	Putative NAD-dependent aldehyde dehydrogenase
GG0919	Putative NADH:flavin oxidoreductase/NADH oxidase
GG0924	Putative 2-nitroprophane dioxygenase
GG0936	Putative UDP-glucosyltransferase
GG1003	Putative UDP-glucosyltransferase
ML013	CYP94A4: Cytochrome P450-dependent fatty acid hydroxylase
ML014	CYP71A8: Cytochrome P450
ML029	Hypothetical protein, esterase
ML118	CYP71AU1: Cytochrome P450
ML222	AER
MT004	Putative NAD-dependent mannitol dehydrogenase
MT019	Bet_v_l motif containing protein
MT042	Aldo/keto reductase like-protein
MT123	Putative 1-aminocyclopropanecarboxylic acid oxidase.
MT173	Putative cytochrome P450
MT175	Cytochrome P450 (MtCYP93E2)

MT178	Putative progesterone 5-beta-reductase
MT187	Putative glycosyltransferase
MT202	Putative progesterone 5-beta-reductase
MT260	Putative alpha-dioxygenase
MT308	Putative NAD-dependent mannitol dehydrogenase
MT309	Putative dioxygenase protein
PG0012	Berberine bridge enzyme-like protein [*Arabidopsis thaliana*]
PG0023	Monoterpene glucosyltransferase [*Eucalyptus perriniana*]
PG0095	SAM (and some other nucleotide) binding motif [*Medicago truncatula*]
PG0105	Glycoside hydrolase, family 1 [*Medicago truncatula*]
PG0119	FAD linked oxidase, N-terminal; Berberine/berberine-like [*Medicago truncatula*]
PG0221	Beta-carotene hydroxylase [*Coffea canephora*]
PG0313	Aldo/keto reductase family protein [*Arabidopsis thaliana*]
PG0317	Putative anthocyanidin-3-glucoside rhamnosyltransferase
PG0408	Dammarenediol-II synthase
PG0525	FAD-binding domain-containing protein [*Arabidopsis thaliana*]
PG0536	2OG-Fe(II) oxygenase [*Medicago truncatula*]
PG0555	CYP71AT2v2 [*Nicotiana tabacum*]
PG0558	Unknown [*Panax ginseng*]
PG0698	AER [*Nicotiana tabacum*]
PG0714	Anthocyanidin 3-O-glucosyltransferase (Flavonol 3-O-glucosyltransferase 1)
PG0746	Hypothetical protein [*Vitis vinifera*]
PG0821	Hydroxycinnamoyl CoA quinate transferase [*Nicotiana tabacum*]
PG1038	Putative sulfolipid synthase [*Oryza sativa* (japonica cultivar-group)]
PG1050	Hypothetical protein [*Vitis vinifera*]

Table 2 shows that 54 constructs were successfully introduced to *M. lanceolata*. However, the growth characteristics of these lines were highly variable with many lines growing extremely slowly. 17 constructs did not generate hairy roots. Fast growing lines were subcultured into liquid medium for analysis. For each overexpression construct, hairy root of a minimum of five independent lines were selected. The obtained hairy roots were screened via the visible GFP-marker (Fig. 3) and RT-PCR for the presence of the transgene. As such, for all of the 8 genes, we obtained at least three independent hairy root lines expressing the transgene.

Table 2 The efficiency of hairy root transformation. For each OE construct, the transgenic hairy root lines were selected based on their GFP expression and confirmed by RT-PCR. The number of fast growing lines is included to show that many lines were growing very slow.

Construct	Number of hairy root lines	Number of fast growing lines	Number of hairy root lines analyzed by RT-PCR	Number of transgenic lines
BF0137	2	-	-	-
BF0232	-	-	-	-
BF0567	-	-	-	-
BF0616	19	16	6	1
BF0838	10	9	3	3
BF0949	4	-	-	-
BF1507	17	8	4	4
BF1557	-	-	-	-
GG0018	5	2	-	-
GG0043	-	-	-	-
GG0048	11	8	5	-
GG0057	9	2	-	
GG0089	33	29	6	3
GG0142	-	-	-	-
GG0167	1	-	-	-
GG0242	15	12	3	1
GG0244	2	-	-	-
GG0347	3	2	2	1
GG0411	7	6	6	1
GG0426	1	-	-	-
GG0436	-	-	-	-
GG0452	-	-	-	-
GG0514	1	-	-	-
GG0526	-	-	-	-
GG0551	2	2	2	-
GG0628	-	-	-	-
GG0739	1	-	-	-
GG0791	1	-	-	-
GG0818	1	-	-	-
GG0888	1	-	-	-
GG0918	-	-	-	-
GG0919	2	-	-	-
GG0924	2	-	-	-
GG0936	2	2	2	-
GG1003	1	-	-	-
ML013	1	-	-	-
ML014	1	-	-	-
ML029	3	1	-	-
ML118	5	1	-	-
ML222	9	5	3	1
MT004	9	9	6	4
MT019	2	1	1	1
MT042	1	-	-	-
MT123	1	-	-	-
MT173	1	1	1	-

MT175	13	10	10	2
MT178	10	10	5	1
MT187	1	1	1	-
MT202	9	7	7	3
MT260	42	35	6	4
MT308	9	1		-
MT309	9	9	5	1
PG0012	7	5	5	1
PG0023	-	-	-	-
PG0095	1	1	-	-
PG0105	-	-	-	-
PG0119	-	-	-	-
PG0221	1	-	-	-
PG0313	5	5	3	3
PG0317	5	-	-	-
PG0408	8	3	3	1
PG0525	2	1	1	-
PG0536	1	-	-	-
PG0555	4	4	4	-
PG0558	-	-	-	-
PG0698	10	10	6	3
PG0714	-	-	-	-
PG0746	7	4	4	1
PG0821	-	-	-	-
PG1038	1	0	-	-
PG1050	-	-	-	-

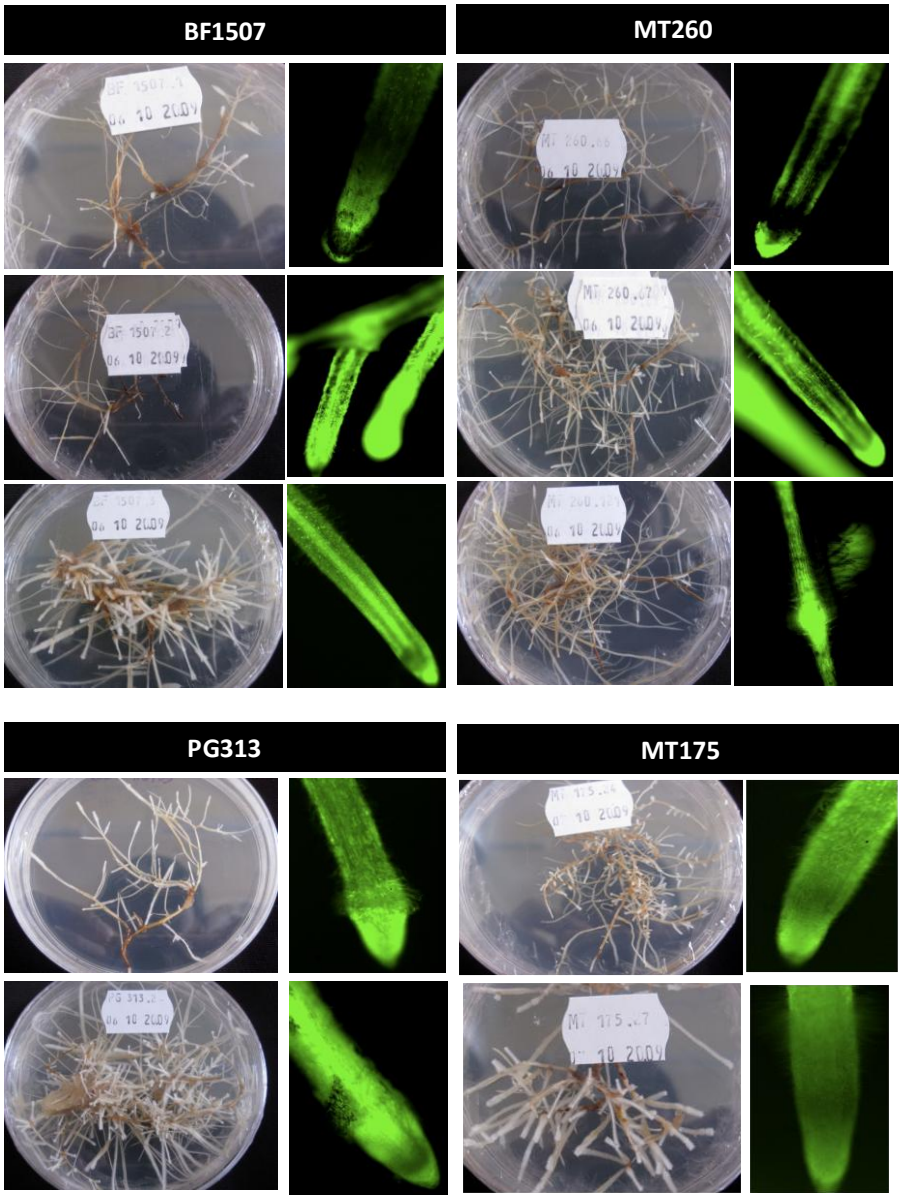


Fig. 3 The respective hairy root phenotypes (left) and GFP expressions (right) of *BF1507*, *MT260*, *PG313* and *MT175* overexpression lines.

In order to check transgene expression level, RT-PCR analysis was done using a subfraction of the upscaled hairy root material. This would allow the comparison between the chemical analysis and the transcript level. We also attempted to find a correlation between the slow growth characteristic and the presence of high transgene transcript levels. Fig. 4 shows an overview of RT-PCR analysis of roots that emerged from leaf disc transformation experiments. Based on RT-PCR results, we concluded that there was no correlation between growth of the lines and the expression of the construct.

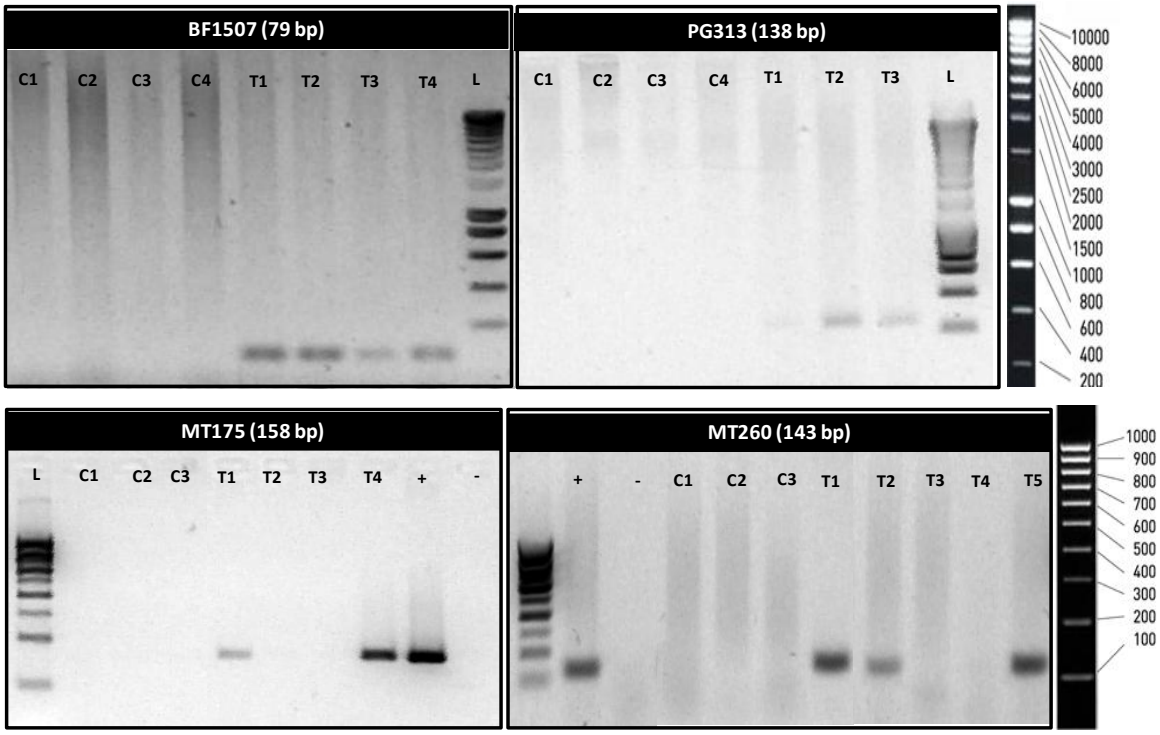


Fig. 4 RT-PCR screening of *M. lanceolata* hairy roots for the presence of transgene in *BF1507*, *PG313*, *MT175*, and *MT260* overexpression lines. C1-C4, control lines; T1-T5, transgenic lines; L, DNA ladder (SmartLadder, Eurogenetec), the size of the ladder fragments are indicated on the right. The cDNA-AFLP fragment and water were used as positive and negative controls respectively.

Saponin analysis

HPLC-MS analysis

The measurements of saponins were performed using HPLC-MS in the Lab of Pharmacognosy and Pharmaceutical Analysis, University of Antwerp (PP-UA). Large variations were measured between different lines containing the same construct (Fig. 5). In addition, no substantial increase was observed in the amount of any of the individual maesasaponins (Fig. 6). Therefore, the production of saponin in these hairy roots must be unstable or alternatively, the method for saponin concentration analysis was not sufficiently robust to allow a comparison. Keeping this variation in mind, we concluded that there were no major changes in saponin content of the overexpression lines compared to the control hairy roots.

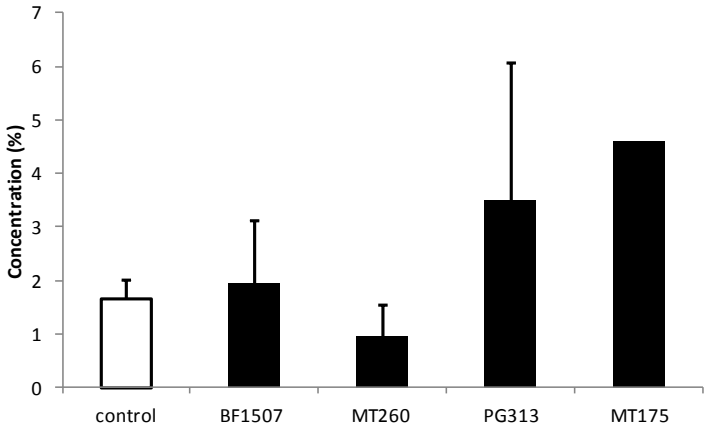


Fig. 5 Total concentration of maesasaponins present in different transgenic lines of *M. lanceolata* hairy roots overexpression lines. Error bars represent standard deviation from the mean of control (n=3), *BF1507* (n=3), *MT260* (n=3), *PG313* (n=2) and *MT175* (n=1).

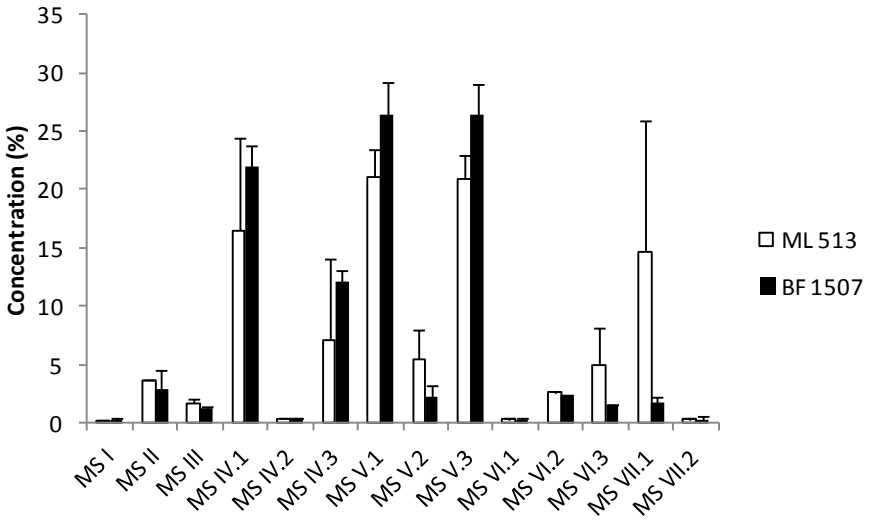


Fig. 6 Concentration of the individual saponins, compared to the total amount of saponin, in control hairy roots (ML513) and in hairy roots of *M. lanceolata* containing an overexpression cassette for a lypolytic enzyme from *B. falcatum*. Error bars represent standard deviation from the mean of triplicate measurements.

ESI-FT-ICRMS analysis

A liquid chromatography (LC) and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICRMS) method for detection of novel compounds in *Maesa* has been developed at PSB-UG/VIB (Pollier et al., 2011). Fig. 7 depicts the UPLC chromatogram of an extract of control *Maesa* hairy roots and Table 2 gives an overview of the masses of the most abundant saponins under the main peaks.

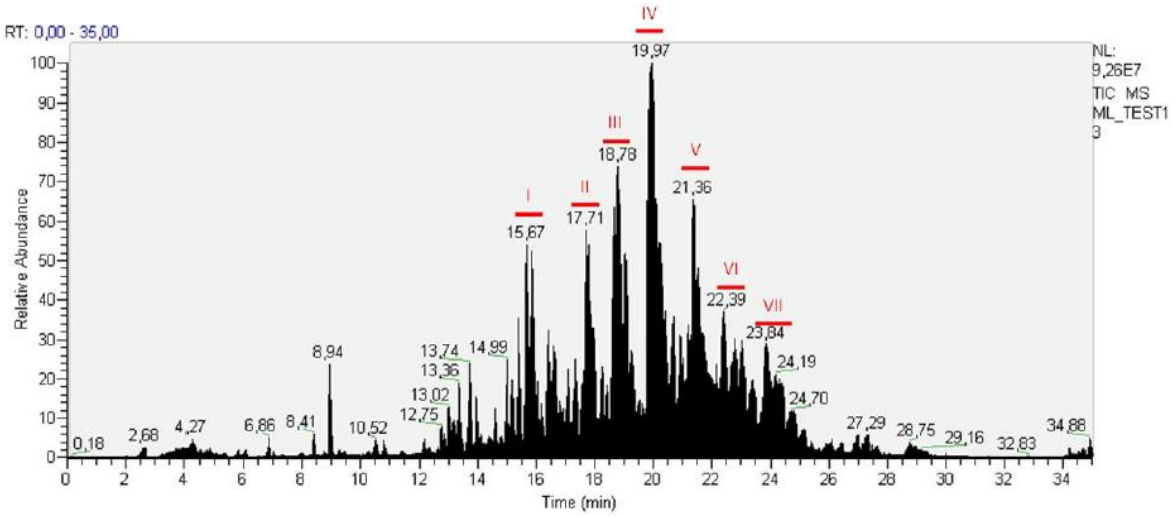


Fig. 7 Chromatogram of the total extract of a control *Maesa* hairy root sample. The 7 main saponin peaks (Maesasaponin I – Maesasaponin VII) are indicated on the chromatogram.

Fig. 7 shows, and also listed in Table 3, that numerous saponins are present in *M. lanceolata* hairy roots which were uncharacterized. Ten out of the 15 reported maesasaponins can be detected in the hairy roots, but it seems there is at least for some of them, more than one isoform of the saponin is present, i.e. 2 or more peaks with the same mass, but with a different retention time. Whether this is an indication of glycosylation or acylation on different positions still needs to be determined. However, based on this analysis, it became clear that *M. lanceolata* contains a complex pool of different saponins, far more than previously assumed, similar to what has been reported for other saponin producing plants such as *M. truncatula* (Pollier et al., 2011).

In order to verify the different methods available, a direct comparison of saponin detection between *Maesa* extracts obtained using an SPE-column and the control mixture (MC3B1) was carried out with FT-MS. In the MC3B1 mixture, 14 of the 15 described saponins could be detected and in the hairy root extracts obtained by an SPE-column, 8 of the 15 saponins were found. Furthermore, in the MC3B1 mixture, the presence of at least 14 additional saponins was shown and in the hairy root extracts at least 46 different saponin molecules were present. Hence, the developed UPLC/ICR-FT-MS method can be considered valid for analysis of *Maesa* hairy root extracts in the future.

Table 3 Saponins present in the *M. lanceolata* control hairy root extract. The masses are ordered according to relative abundance under the peak of the UPLC chromatogram.

Peak	Main Masses (Da) $[M-H]^-$	Saponin
Peak I	1233,60	Maesasaponin I
	1235,61	
	959,53	Saponin with only 3 sugars (3x Hex)
	1105,59	
	1177,57	
	1298,59	
	1221,60	
	783,49	Saponin with only 2 sugars (2x Hex)
	1249,60	
Peak II	1263,61	
	1103,57	
	1338,62	
	1191,55	
	1381,55	
	1291,61	
Peak III	1277,62	
	1275,61	Maesasaponin III.2
	1205,61	
	1305,62	Maesasaponin III.1
	941,48	
	1087,54	
Peak IV	1219,62	
	1217,61	
	1289,63	Maesasaponin IV.3
	1291,64	
	1319,64	
	1239,59	
	1247,62	Maesasaponin IV.1
Peak V	1231,62	
	1303,64	Maesasaponin V.3
	1087,58	
	1265,61	
	1259,62	Maesasaponin V.1
	1325,63	
	1337,63	
Peak VI	1315,64	Maesasaponin VI.2
	1317,65	
	1345,65	Maesasaponin VI.3
	1337,63	
	1343,64	
	1339,64	
	1313,63	
Peak VII	1329,66	
	1357,65	Maesasaponin VII.1
	1363,64	
	1327,65	
	1355,64	
	1447,60	
	1153,59	
	1379,64	
	1475,59	

Combinatorial biosynthesis using MT175 (a cytochrome P450)

MT175 is annotated as CYP93E2, a CytP450 from *M. truncatula*. CYP93E2 is a homolog of CYP93E1 responsible for β-amyrin 24-hydroxylase activity resulting in 24-hydroxy-β-amyrin (Fig. 8) in *Glycine max* (Shibuya et al., 2006) and in the legume *Glycyrrhiza uralensis* (Seki et al., 2008). Since all known saponin from *M. lanceolata* are derived from β-amyrin and oxidation occurs on a different position of the backbone, overexpression of MT175 in *M. lanceolata* may lead to the production of new compounds to both parental species.

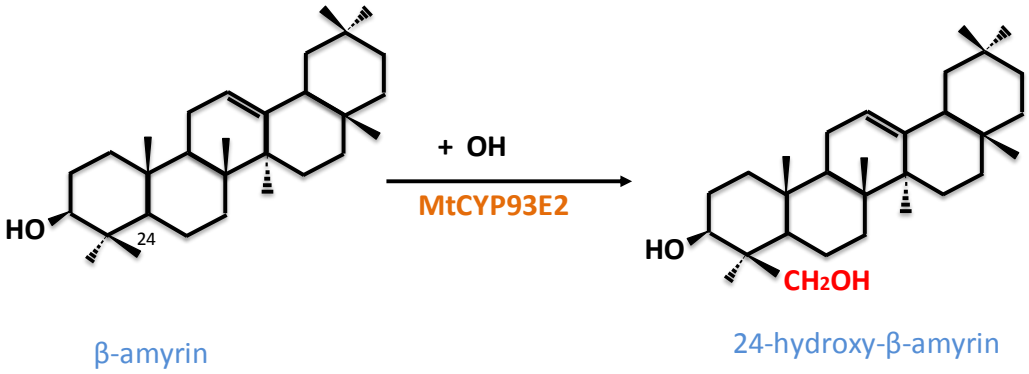


Fig. 8 Hydroxylation of β-amyrin skeleton at position C24 by Mt175 (MtCYP93E2)

After confirmation of overexpression with RT-PCR (Fig. 4), 5 biological repeats of 2 independent transgenic lines and 3 control lines were profiled with liquid chromatography electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (LC-ESI-FT-ICRMS) to compare saponin composition of overexpression roots with that of control roots. Comparative analysis of the control root extract and MT175 overexpression roots yielded a total of 8,389 *m/z* peaks. Furthermore, 85 discrete peaks between MT175 overexpression roots and control roots were observed, corresponding to 3 unique compounds. These compounds were present in all of the MT175 overexpression lines and were absent in all of the control lines, potentially representing compounds with a hybrid structure.

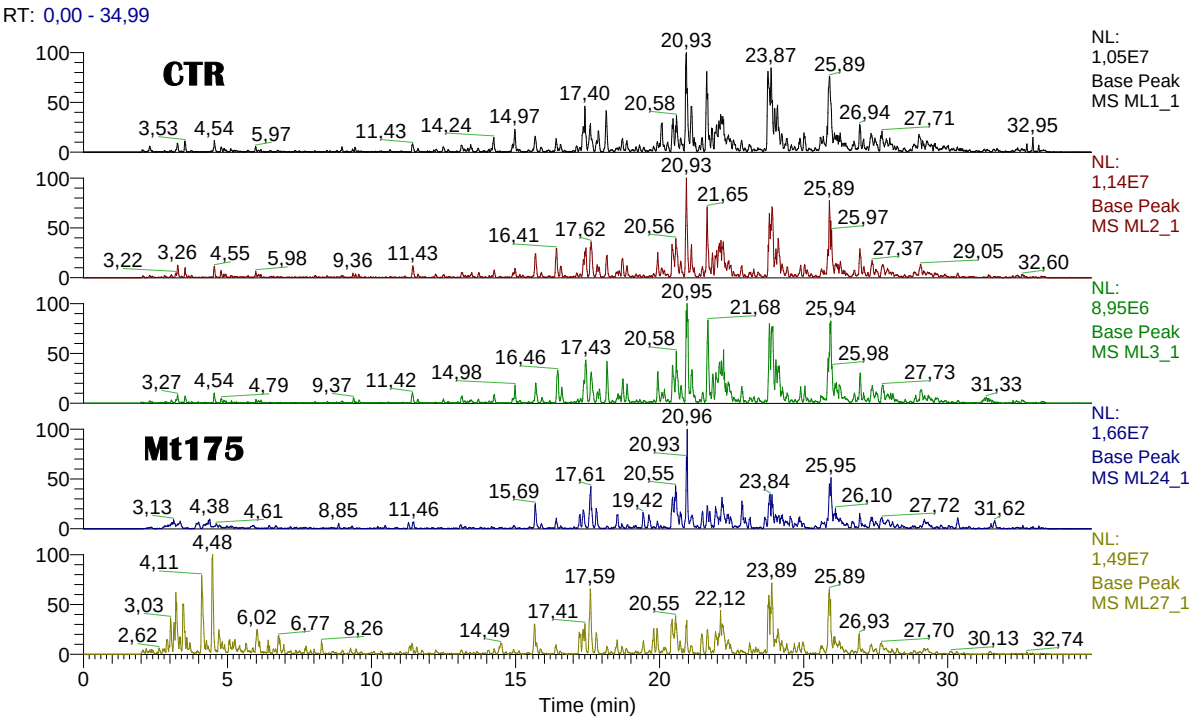


Fig. 9 Metabolite profile of control (CTR) hairy roots (n=3) and MT175 hairy root lines (n=2).

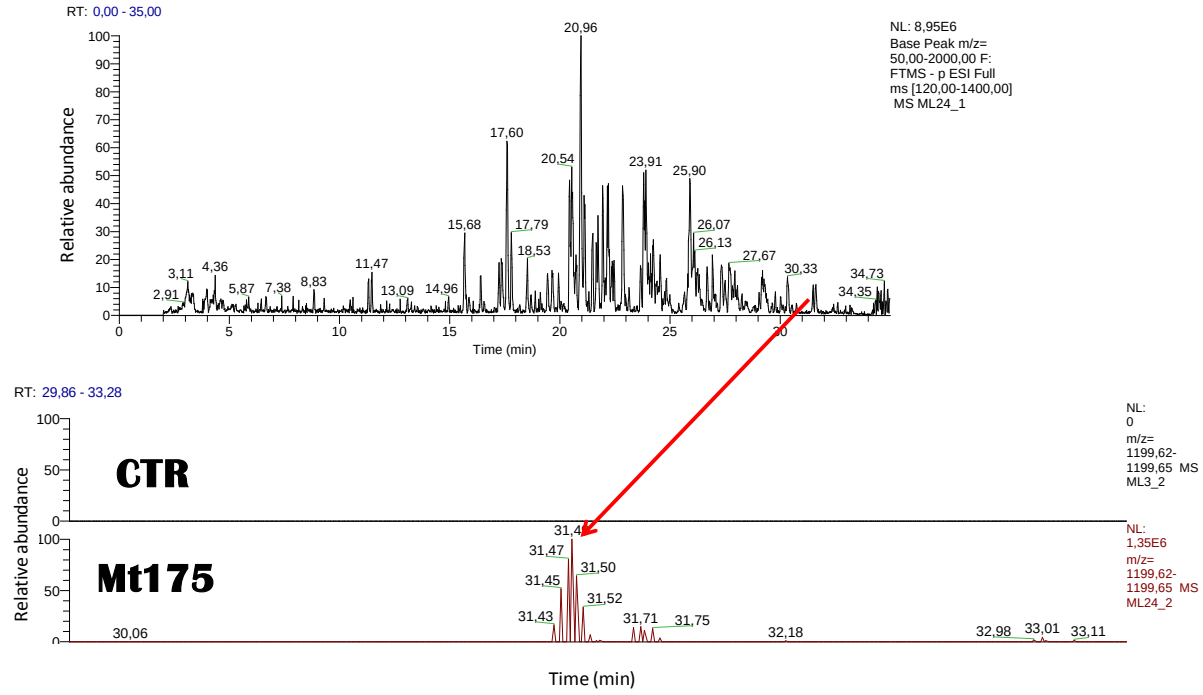


Fig. 10 Candidate novel compound which is abundant in MT175 overexpression lines, but absent in hairy root control (CTR).

The tentative identification of the saponins was obtained by MS^n fragmentation using the linear ion trap (IT). Full FT-MS spectra were interchanged with dependent IT-MS^n

scan events, consisting of one MS^2 and two MS^3 scans. In the MS^2 scan, the most abundant ion in the previous full MS scan was fragmented. The two most abundant daughter ions in every MS^2 scan event were subjected to a MS^3 scan event. As such, for some of the most abundant new compounds, the aglycone backbone could be determined. For instance, the new compound at retention time (R_T) 31.41 min yielded a $[M-H]^-$ anion at *m/z* 1199.63 (Fig. 11). The sugar residues and the aglycone were identified from the MS^n spectra. For this saponin MS^2 fragmentation led to the generation of daughter ions, the smallest of which, at *m/z* 553, represents the aglycone ion, $[Agly-H]^-$ (Fig. 12a).

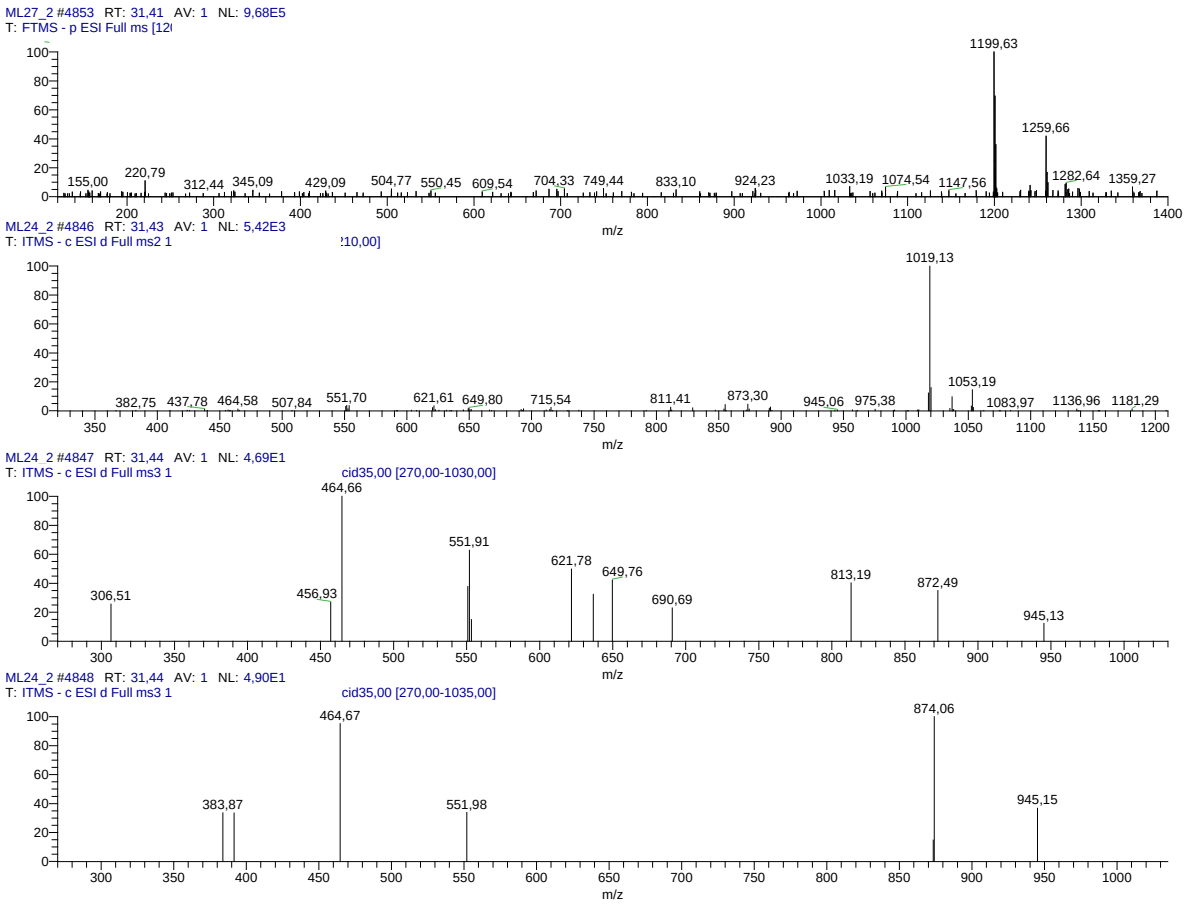


Fig. 11 LC ESI FT-ICR MS chromatograms of MT175. (a) MS scan of peak at R_T 31.44 min. (b) MS^2 fragmentation of the $[M-H]^-$ ion at *m/z* 1199.63 (c) MS^3 fragmentation of the most abundant MS^2 daughter ion of the $[M-H]^-$ ion at *m/z* 1199.63

As such, we could identify two additional new aglycones with the $[Agly-H]^-$ anion at *m/z* 539 (Fig. 12b) and *m/z* 595 (Fig. 12c) respectively. These aglycones showed the characteristic MS/MS pattern of maesasaponins. Unfortunately, we were not able to

propose a structure for the observed aglycones. Surprisingly, although absent in the control lines, these compounds also occurred in other lines carrying the overexpression cassettes BF1507, MT260, and PG313. As these cassettes do not contain the MT175 open reading frame, there is not strict correlation between MT175 expression and the novel compounds. Therefore, the detection of novel aglycones was not the result of the combinatorial biosynthesis, but rather a variation in plant materials, from which hairy roots were derived or variation evoked by transformation process or culturing conditions. Currently, it is unclear what factor may have caused the variability in the detection of putative saponin.

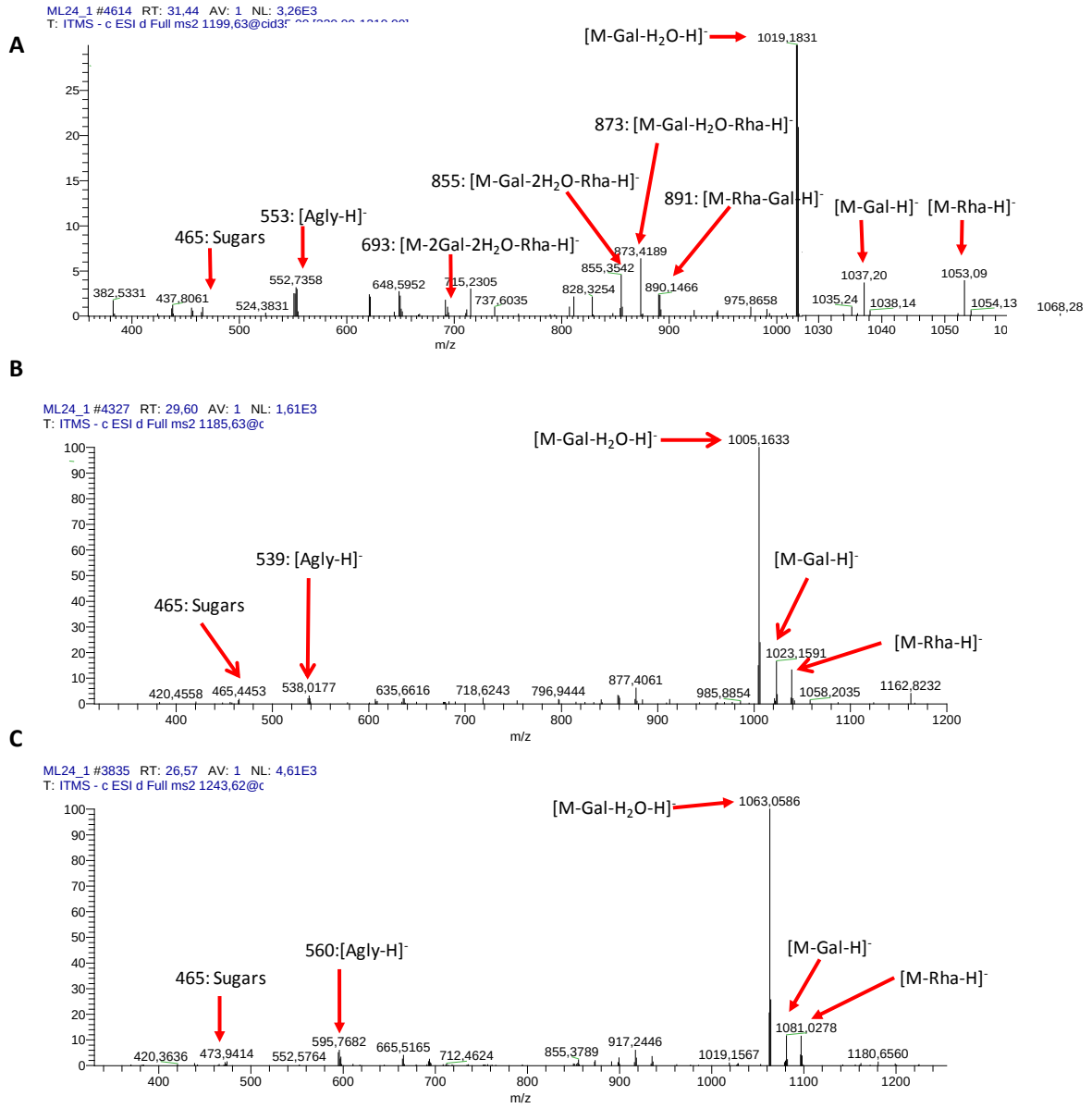


Fig. 12 MS^2 fragmentations of (a) the $[M-H]^-$ ion at *m/z* 1199.63 (R_T = 31.44 min), (b) the $[M-H]^-$ ion at *m/z* 1185.63 (R_T = 29.60 min), and (c) the $[M-H]^-$ ion at *m/z* 1273.62 (R_T = 26.57min) lead to identification of new aglycones with the $[Agly-H]^-$ anion at *m/z* 553, 539, and 560 respectively.

3. Discussion

An efficient method for transformation using *A. rhizogenes* was established in *M. lanceolata*. Transgenic hairy roots were induced on *Maesa* leaf discs and were grown on culture medium without the need for exogenous hormones. TLC analysis was used to detect saponins in *M. lanceolata* hairy roots that were qualitatively and quantitatively comparable to normal roots of greenhouse grown and in vitro grown plants (Lambert, 2011). Hairy roots are more differentiated than cell cultures, therefore, they are genetically and biochemically more stable and consequently offer a promising alternative for the production of important metabolites (Georgiev et al., 2007). For *M. lanceolata*, however, the growth characteristics of its hairy root lines were highly variable with many lines growing extremely slow. Additional analysis with RT-PCR also showed that some of overexpressed genes were not detected in hairy root lines, although they showed positive signal of GFP fluorescence. This was probably a result of the partial incorporation of T-DNA genes into the host genome. As previously reported, the presence or absence of several T-DNA genes responsible for hairy root induction including auxin and opine synthesis was shown to influence morphology, growth, biosynthetic gene expression and metabolite accumulation in *Catharantus roseus* hairy roots (Taneja et al., 2010).

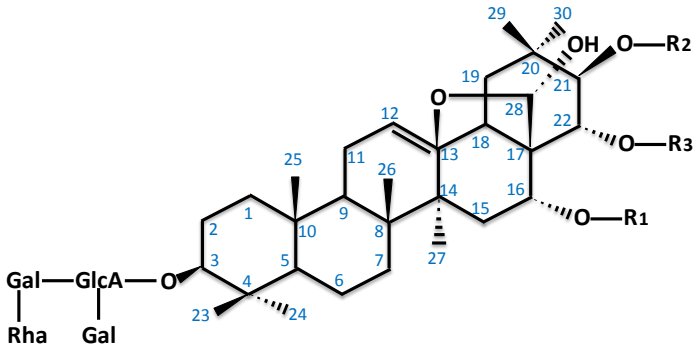


Fig. 13 Saponin from *M. lanceolata*

Saponins produced by *M. lanceolata*, namely maesasaponins (Fig. 13) are an oleanane-derived triterpenoid saponins distinguished by their characteristic C13,28 hemiacetal or ester bridges, which result from the reaction between a C-13 hydroxyl and C-28 aldehyde or carboxyl group. Additionally, maesasaponins constitute a structurally consistent series of mono-, di- and trimesters, in which C-21 always substituted with an

angeloyloxy group, C-16 can be substituted with an acetoxy group, and C-22 with a variable acyloxy group (acetoxy, propanoyloxy, butanoyloxy or angeloyloxy) (Sindambiwe et al., 1996).

An alternative approach to generate novel chemical structures is combinatorial biosynthesis, in which genes from different organisms are introduced in an organism in the hope that the combined enzymatic activity leads to new chemical structures and possibly new bioactive molecules. This approach has been widely applied in microorganisms and has been shown to be possible in plants as well (reviewed by Polier et al., (2011)). In this study, we used combinatorial biosynthesis to create novel maesasaponin molecules. To this end, the full length-open reading frames corresponding to cDNA-AFLP tags were cloned and introduced in overexpression cassettes. The misexpression of saponin biosynthesis gene was anticipated to generate novel substitution groups or to change the skeleton of maesasaponins and consequently, novel molecules with potentially modified properties could have been created. In total 54 gene constructs from 4 different plant species were transformed in *M. lanceolata*. Genes were isolated from *B. falcatum*, *M. truncatula*, *P. ginseng* and *G. glabra.* These plant species produce structurally related triterpene saponins, however, with different structural features linked to different biological activities.

In our genome-wide transcript profiling we identified a cytchrome P450 (MT175) that was annotated as CYP93E2, a CytP450 responsible for β-amyrin 24-hydroxylase activity in *M. truncatula*. Therefore, the overexpression of this gene in *M. lanceolata* became a top priority to create hairy root lines for the identification of novel molecules as a Combiplan proof of concept. Five repeats of each 3 independent confirmed overexpression hairy root lines were analysed with HPLC-MS techniques (more specifically UPLC/ICR-FT-MS or Ultrahigh Performance Liquid Chromatography/Ion Cyclotron Resonance – Fourier Transform – Mass Spectrometry). From this construct, 3 unique peaks were found in the chromatogram of all overexpression repeats that were not observed in the control lines. Unfortunately, these peaks were also present in other overexpression lines transformed with different constructs. Therefore, these compounds are not the result of a combinatorial process, but rather an altered regulation of the steady state pathway. Further research is required to explain the gained capacity to produce novel saponins.

4. Materials and methods

M. lanceolata hairy root induction and culture

M. lanceolata hairy roots were induced through *A. rhizogenes* (strain LBA 9402/12) transformation on leaf discs. The *Agrobacterium* strain was transformed with the pK7WG2D plasmid (Fig. 14). *Agrobacteria* were transferred from a glycerol stock to 3 mL of liquid YEB medium. This medium contained antibiotics (100 mg/L rifampicin (Sigma), 100 mg/L spectinomycin (Sigma) and 300 mg/L streptomycin (BDH)) and consisted of 5 g/L beef extract (Sigma), 1 g/L yeast extract (Duchefa, The Netherlands), 5 g/L peptone (Duchefa, The Netherlands), 0.15 M sucrose and 2 mL/L of 1 M $MgSO_2.7H_2O$. The bacterial cultures were incubated on a rotary shaker (220 rpm) at 28 °C. After 48 h, 45 µL of this bacterial preculture was transferred into 15 mL of fresh YEB medium with the same antibiotic concentrations mentioned above. The bacterial cultures were again incubated on a rotary shaker (220 rpm) at 28 °C. After 24 h, these cultures were used for the transformation. For selection of the transgenic material, GFP was used as a visible marker.

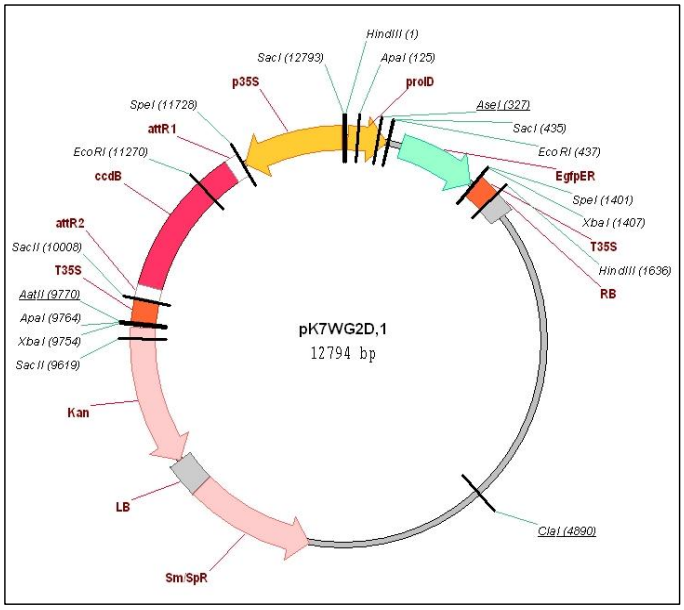


Fig. 14 Vector backbone for gene overexpression (pK7WG2D) in *M. lanceolata* hairy roots

For hairy root induction, in vitro leaf material was wounded and co-cultivated with the *Agrobacteria*. The co-cultivation medium consisted of MS salts with vitamins supplemented with 0.8% (w/v) agar, 2% (w/v) sucrose and 4 µM benzyladenine (BA) (Sigma).

After 3 days, the leaves were transferred to cultivation medium comprising MS salts with vitamins, 0.8% (w/v) agar, 2% (w/v) sucrose and 500 mg/L cefotaxime (Duchefa, The Netherlands) to arrest bacterial growth. Hairy roots were isolated from the leaf material after 4-6 weeks and were placed on solid Schenk and Hildebrandt (SH) medium including vitamins (Schenk and Hildebrandt, 1972) and supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose. The cultures were incubated in the dark at 25 °C and were monthly subcultured.

For saponin analysis, hairy roots were grown in liquid cultures. Hairy roots were transferred to Erlenmeyer flask with 10 ml of the SH culture medium described above.

Reverse transcription PCR (RT-PCR)

M. lanceolata hairy roots grown for 4 weeks in liquid medium were harvested and rinsed with water. Subsequently, the roots were frozen and ground in liquid nitrogen. For RT-PCR, total RNA was extracted with Concert™ Plant RNA Reagent (Invitrogen), and cDNA was prepared with SuperScript^{TM} II Reverse Trancriptase (Invitrogen). Specific PCR primers were designed based on the cDNA-AFLF tag sequences with the Primer3 program (Rozen and Skaletsky, 2000).

Metabolite extractions

Metabolite extraction for *M. lanceolata* was adapted from protocol developed for *M. truncatula* (Pollier et al., 2011). Harvested *M. lanceolata* hairy roots were frozen and ground in liquid nitrogen. Of the ground material, 400 mg was extracted with 1 mL methanol at room temperature for 10 min, followed by centrifugation for 10 min at 14000 rpm. Under vacuum, 750 µL of the supernatant was evaporated to dryness and residue was dissolved in 400 µL water and 200 µL cyclohexane. Samples were then centrifuged for 10 min at 14000 rpm and 200 µL of the aqueous phase was retained for analysis.

Supporting work

Elicitation, AFLP, gene sequencing and selection

Initial experiments of the Combiplan were performed by the Secondary Metabolites Group from the Department of Plant Systems Biology (VIB).

For elicitation, *in vitro* shoots of *M. lanceolata* were sprayed with 500 µM MeJA or ethanol (as a control) until run-off. Leaves were harvested 0, 0.5, 1, 2, 4, 8, 24 and 48 hours after elicitor treatment. After sampling, total RNA was prepared with TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed to double-stranded cDNA as described (Vuylsteke et al., 2007). Upon appropriate sample preparation, cDNA-AFLP based transcript profiling was performed as described with all 128 possible BstYI+1/MseI+2 primer combinations (Rischer et al., 2006; Vuylsteke et al., 2007). Gel images were analyzed with the AFLP-QUANTARPRO software (Keygene, Wageningen, The Netherlands) allowing accurate quantification of the band intensities. The intensity of all individual bands was determined and the obtained raw expression data were corrected for lane variations (due to PCR or loading differences) by dividing the raw data by a correction factor. The correction factor was calculated by dividing the sum of the expression levels of all fragments within one lane by the highest sum of all lanes within a primer combination. Subsequently, the standard deviation (SD) and the average were calculated for each individual band. Individual gene expression profiles were variance normalized by subtracting the calculated average from each individual data point, after which the obtained value was divided by the SD. A coefficient of variation (CV) was obtained by dividing the SD by the calculated average. Gene tags displaying expression values with a CV$\geq$0.5 were considered as differentially expressed. Based on this cut-off value, together with visual inspection of cDNA-AFLP gels, differentially expressed gene tags were selected for further analysis. Cluster analysis, sequencing and BLAST analysis were performed as described by Rischer et al. (2006).

Quantitative measurement by HPLC-MS analysis

All saponin analyses were performed in the Lab of Pharmacognosy and Pharmaceutical Analysis (University of Antwerp). The protocol for *Maesa* sample preparation and HPLC-MS is described in Theunis et al. (2007).

Metabolic profiling by LC ESI FT-ICR MS

Metabolite profiling using LC ESI FT-ICR MS was performed in PSB-UG/VIB and the protocol is described in Pollier et al. (2011). For reversed-phase LC, an Acquity UPLC BEH C18 column (150 x 2.1 mm, 1.7 μm; Waters, Milford, MA) was serially coupled to an Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 μm) and mounted on an ultra-high-performance LC system consisting of a Accela pump (Thermo Electron Corporation, Waltham, MA, USA) and Accela autosampler (Thermo Electron Corporation). The Accela LC system was hyphenated to a LTQ FT Ultra (Thermo Electron Corporation) via an electrospray ionization source. The following gradient was run using water:MeCN (99:1, v:v) acidified with 0.1% (v:v) HOAc (solvent A) and MeCN:water (99:1, v:v) acidified with 0.1% (v:v) HOAc (solvent B): time 0 min, 5% B; 30 min, 55% B; 35 min, 100% B. The loop size, flow, and column temperature were 25 µL, 300 µL/min and 80°C, respectively. Full loop injection was applied. Negative ionization was obtained with the following parameter values: capillary temperature 150 °C, sheath gas 25 (arbitrary units), aux. gas 3 (arbitrary units), and spray voltage 4.5 kV. Full FT-MS spectra between *m*/*z* 120–1400 were recorded at a resolution of 100,000. For identification, full MS spectra were interchanged with a dependent MS^2 scan event in which the most abundant ion in the previous full MS scan was fragmented, and two dependent MS^3 scan events in which the two most abundant daughter ions were fragmented. The collision energy was set at 35%.

Acknowledgment

We thank Prof. Alain Goossens for providing the overexpression constructs, Jacob Pollier for metabolite profiling with FT-MS and helpful discussion with combiplan data, and Kenn Foubert for HPLC measurements. A.F. is supported by the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia and the BOF (special research fund No. 01WI0111, Ghent University).

5 | Agroinfiltration of intact leaves as a method for the transient and stable transformation of saponin producing *Maesa lanceolata*

Cover art: Tobacco nuclei | Inverted microscopy image of tobacco leaf nuclei expressing NLS-GFP

5

Agroinfiltration of intact leaves as a method for the transient and stable transformation of saponin producing *Maesa lanceolata*

Published in:

Ahmad Faizal and Danny Geelen. **Agroinfiltration of intact leaves as a method for the transient and stable transformation of saponin producing *Maesa lanceolata***. *Plant Cell Reports* (2012), 31: 1517-1526

Abstract

A method has been developed to genetically transform the medicinal plant *Maesa lanceolata*. Initially we tested conditions for transient expression of GFP-bearing constructs in agroinfiltrated leaves. Leaf tissues of *M. lanceolata* were infiltrated with *Agrobacterium tumefaciens* carrying a nuclear-targeted GFP construct to allow the quantification of the transformation efficiency. The number of transfected cells depended on the bacterial density, bacterial strains, the co-cultivation time, and presence of acetosyringone. The transient transformation assay generated the highest ratio of transfected cells over non-transfected cells upon 5 days post-infiltration using *A. tumefaciens* strain LBA4404 at an OD_{600} = 1.0 in the presence of 100 µM acetosyringone and in the absence of a viral suppressor construct. In a second series of experiments we set up a stable transformation protocol that resulted in the regeneration of kanamycin-resistant plants expressing nuclear GFP. This transformation protocol will be used to introduce overexpression and RNAi constructs into *M. lanceolata* plants that may interfere with triterpenoid saponin biosynthesis.

1. Introduction

Maesa lanceolata is used in African traditional medicine, whereby extracts of leaves and fruits are used for the treatment of various diseases including hepatitis, dysentery, skin diseases and neuropathies (Sindambiwe et al., 1996). Metabolic profiling has identified the presence of a mixture of triterpenoid saponin (maesasaponins) which have been tested for pharmaceutical activities (Sindambiwe et al., 1998; Muhammad et al., 2003; Tadesse et al., 2009). Specific subclasses of maesasaponins showed anti-angiogenic activity lacking haemolytic activity, which have a potential for the development of drugs for certain types of cancers (Apers et al., 2002).

Because maesasaponins are highly complex structures, organic synthesis is not feasible for the further biological activity analysis and we therefore need to develop strategies that can modulate saponin biosynthesis in the plant. As part of the characterization of candidate genes we have developed a transformation protocol for hairy root induction. Although hairy root induction is perfectly suitable for the screening of different constructs and analysis of small samples of transgenic material, it may not be suitable for the expression of leaf-derived cDNA sequences. We therefore set up an *Agrobacterium tumefaciens*-mediated transformation protocol. Both a transient expression and stable transformation method were developed. The transient expression system was optimized to allow the screening of candidate genes which are suspect to enhance saponin production or drive production towards one specific class of saponins.

Transient gene expression assays mediated by agroinfiltration have been successfully used as tool for rapid analysis of plant promoters and transcription in tobacco and rice (Yang et al., 2000; Zhang et al., 2012), isolation of disease resistance gene in potato (Bendahmane et al., 2000), and functional analysis of other genes in plant within a few days of infiltration (Hoffmann et al., 2006; Figueiredo et al., 2011; Leckie and Neal Stewart, 2011; Bertazzon et al., 2012). The efficiency of transient gene expression is greatly influenced by the limitation of *A. tumefaciens* virulence and the physiological condition of the plants (Wroblewski et al., 2005). Therefore, an efficient method for transient gene expression and stable transformation in *Maesa* is critical to the study of gene function by overexpression or by gene silencing.

Notably, transient gene expression assays are readily applicable, do not require expensive supplies and equipment, and allow rapid screening of gene activity within leaf tissue (Wroblewski et al., 2005). In addition, it has been shown to work with great effectiveness in several model species, such as Arabidopsis, tobacco, tomato, potato, grapevine and rose (Kościańska et al., 2005; Zottini et al., 2008; Bhaskar et al., 2009; Kim et al., 2009; Yasmin and Debener, 2010; Tsuda et al., 2012).

In this report, we describe the development of a transient expression system for *Agrobacterium*-mediated transformation of *M. lanceolata*. Under optimal transfection conditions, we have been able to develop a stable transformation method that takes advantage of the high transfection rates by directly transferring transfected leaf tissue onto regeneration and cultivation medium.

2. Results

Transient expression upon agroinfiltration of intact leaves

The level of transient expression in an agroinfiltration system depends on the efficiency of transformation and the transcriptional and translational activity of the monitored marker. In preliminary experiments, we tested seven different *Agrobacterium tumefaciens* strains (C58, EHA101, EHA105, LBA4404, GV3301, GV2260 and pMP90), and found that LBA4404 resulted in consistently higher transformations than the other strains (data not shown). The nuclear-targeted GFP reporter (NLS-GFP) carried by *Agrobacterium tumefaciens* strain LBA4404 was highly expressed in transformed leaf cells and allowed rapid and reliable identification of cells that were transformed and expressed the nuclear GFP in infiltrated leaves (Fig. 1). The transient transformation assay was optimized by evaluating the effects of bacterial density, the co-cultivation time, the presence of acetosyringone and the presence of a viral silencing suppressor construct.

The transformation efficiencies were determined by dividing the average of GFP fluorescent nuclei (Fig. 2a) by the average number of DAPI-stained nuclei observed within a transfected area of the same leaf blade (Fig. 2b).

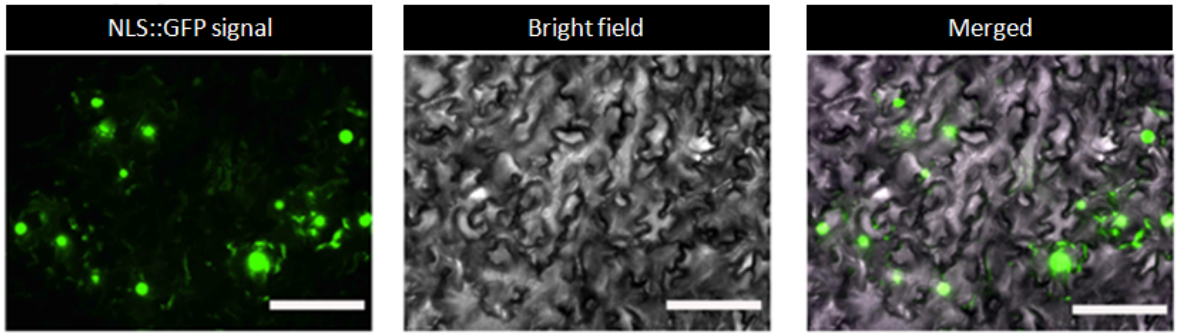


Fig. 1 Microscopy images showing the nuclear-targeted GFP fluorescence from agroinfiltrated leaf tissue. *Bar* = 100 µm.

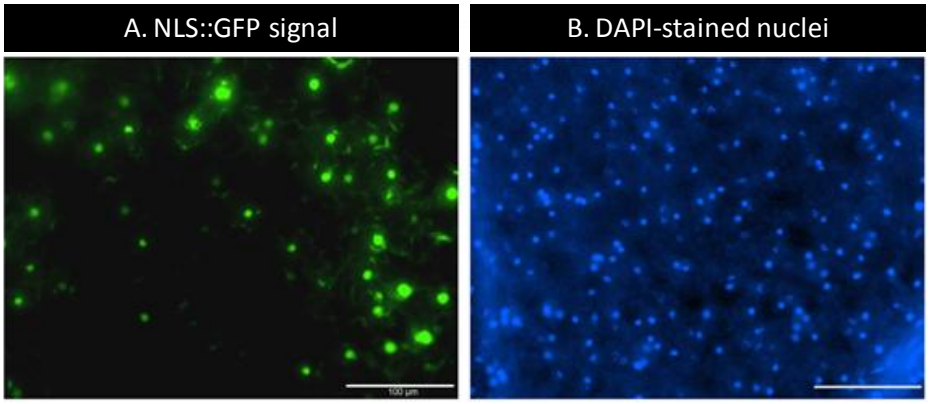


Fig. 2 Microscopy analysis of transfected *M. lanceolata* leaf blades. **a**, NLS-GFP fluorescence showing fluorescent nuclei; **b**, fluorescence from DAPI-stained nuclei at a similar region of the transfected leaf blade. Images were taken with the same magnification. *Bar* = 100 µm.

The bacterial density

Bacterial cell density of *Agrobacterium* suspensions has been shown to be an important factor in transient expression in several plants (Wroblewski et al., 2005; Bhaskar et al., 2009). In Arabidopsis, cell suspensions with a cell density less than OD_{600} = 0.1 often result in low expression and those with cell densities above OD_{600} = 1.0 result in tissue yellowing or wilting (Wroblewski et al., 2005). We tested different cell densities of *A. tumefaciens* LBA4404 of OD_{600} 0.1, 0.3, 0.5, 0.8, 1.0, 1.3, and 1.6. As shown in Fig. 3a, the transient expression level initially increased with the increasing density of *Agrobacterium*. It reached the maximum transient expression level at OD_{600} of 1.0 and then decreased at OD_{600} higher than 1.0.

The co-cultivation time

In the leaf blades we infiltrated, we did not observe plant cell division or substantial leaf expansion during the period of transfection (data not shown). The cells showed transient NLS::GFP expression from the second day post-infiltration (dpi) and the number of cells expressing GFP increased to a maximum at 5 dpi. Afterwards the number of cells expressing GFP as well as the intensity of fluorescence decreased significantly (Fig. 3b).

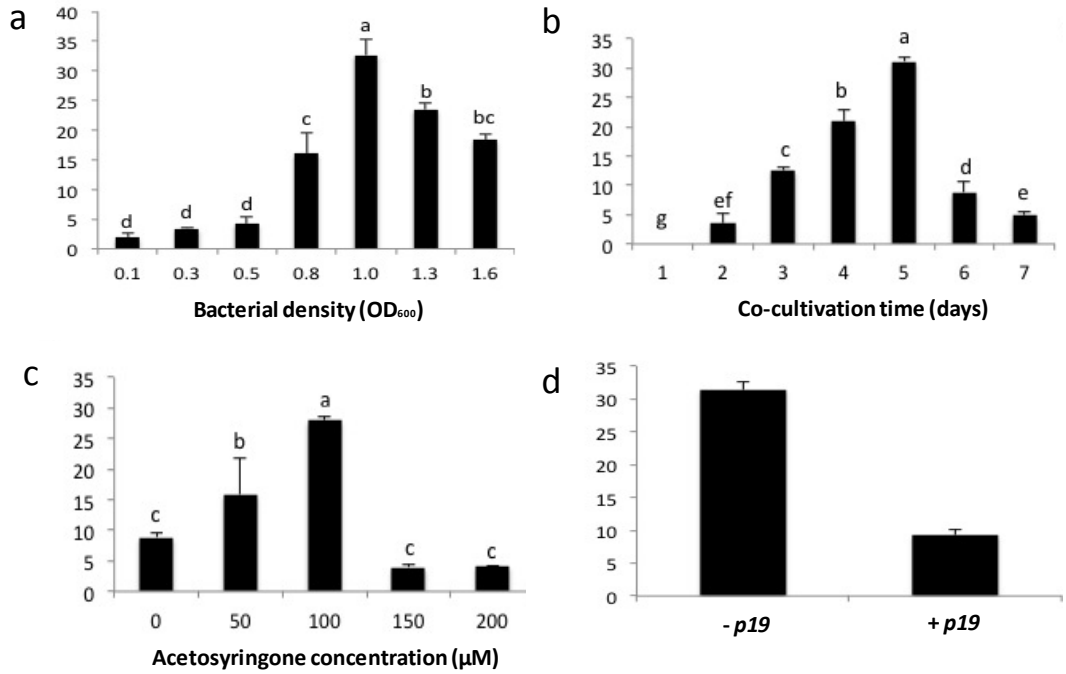


Fig. 3 Frequency of transient GFP expression for the effect of bacterial density (a), days post-infiltration (b), the presence of acetosyringone (c) and co-transfection with *p19* (d). The y-axis indicates the mean frequency of transient GFP expression of each factor. The frequency data were generated with at least 5 replicates and 3 independent repetitions. Different letters above each column indicate significant differences between the mean values at $P < 0.05$.

The presence of acetosyringone

We also investigated the effect of acetosyringone since it has been reported to influence transformation efficiency and transient gene expression in many plant species (Godwin et al., 1991; Kapila et al., 1997). Acetosyringone was added to the infiltration medium at different concentrations. The addition of 50 µM of acetosyringone doubled the transformation efficiency. Furthermore, the transformation efficiency achieved its maximum level when the concentration of acetosyringone was increased to 100 µM.

Concentrations higher than 100 µM acetosyringone had a negative effect on the transformation efficiency (Fig. 3c).

Co-infiltration with a viral silencing suppressor

Dhillon and co-workers reported that co-introduction of plant viral suppressors of gene silencing *HCPro* (from *Tobacco etch virus*), *p19* (from *Tomato bushy stunt virus)*, *γb* (from *Barley stripe mosaic virus*) and *p21* (from *Beet yellows virus*) with GFP on a separate plasmid lead to an almost twofold increase in initial GFP expression levels in *Phaseolus lunatus* (Lima bean) cotyledons (Dhillon et al. 2009). Therefore, we also co-introduced one of the silencing suppressors, *p19* with the GFP gene on a separate plasmid via agroinfiltration and evaluated the effect on transient GFP expression in *M. lanceolata*. In the case that the transient gene assay would be limited by post-transcriptional gene silencing (PTGS), the co-infiltration with *p19* could enhance transfection rates. However, compared to the GFP expression plasmid alone, co-infiltration of the NLS::GFP with *p19* resulted in significantly lower transfection and GFP expression (Fig. 3d).

Stable transformation of *M. lanceolata*

Initial attempts to transform *M. lanceolata* using leaf discs immersed with *Agrobacterium* cultures did not result in transgenic plants. Also, an inspection of the immersed tissue showed that very few of the cells at the cutting edges were transfected. Having established an optimized transient transformation assay, we decided to use infiltrated leaf discs for generating stable *M. lanceolata* transformants. To compare the classic leaf disc immersion technique with the infiltrated leaves we performed experiments in parallel. A schematic view of the two approaches is shown in Fig. 4. The dose of antibiotics required for selection was tested by incubating the leaf discs on shoot induction medium containing different concentration of cefotaxime and kanamycin (Table 1). Kanamycin at 25 mg/L completely inhibited shoot regeneration from leaf explants following a protocol previously described (Faizal et al., 2011). Because leaf explants showed resistance to higher kanamycin concentrations, we used 100 mg/L. At this concentration, kanamycin had a necrotic effect on axillary propagated shoots. This concentration was effective to select stably transformed plants without lethality.

Table 1 The effect of cefotaxime and kanamycin on adventitious shoot regeneration induced from *M. lanceolata* leaf explants incubated for 8 weeks on shoot induction medium (SIM).

Antibiotic (mg/L)		No. of shoot/explant ± SEM
Cefotaxime	Kanamycin	
-	-	3.3 ± 0.7[b]
125	-	2.8 ± 0.8[b]
250	-	8.7 ± 1.4[a]
500	-	10.1 ± 1.3[a]
-	25	0.00
-	50	0.00
-	75	0.00
-	100	0.00

Each petri dish with four explants was considered as one replicate. The presented experiments comprise at least 5 replicates with 3 repetitions. Different letters indicate significant differences between mean values at $P < 0.05$ within each treatment.

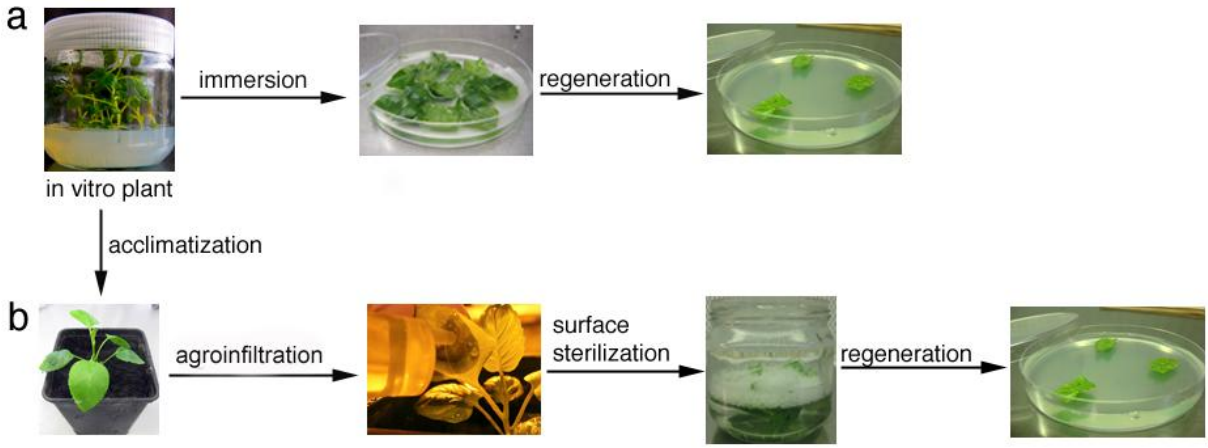


Fig. 4 Schematic diagram of *A. tumefaciens*-mediated transformation (**a**) immersion and (**b**) agroinfiltration method.

The impact of cefotaxime on regeneration capacity was also analyzed. Cefotaxime is usually applied to stop growth and eliminate remaining *Agrobacteria*. The influence of cefotaxime on shoot induction was examined by incubating leaf explants on shoot induction medium containing 0, 125, 250, and 500 mg/L cefotaxime. Cefotaxime at 250 and 500 mg/L had a stimulatory effect and significantly increased the number of adventitious shoots emerging on the leaf explants compared to control leaf explants. Therefore, for the subsequent experiments we used 100 mg/L kanamycin and 250 mg/L cefotaxime for transgenic selection.

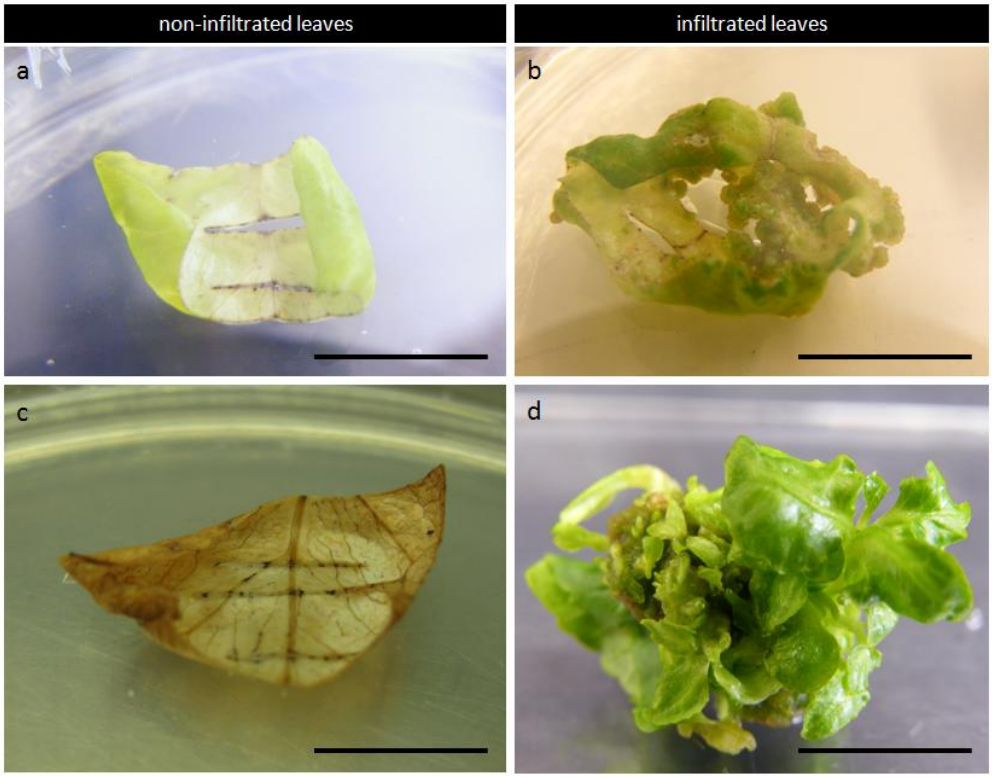


Fig. 5 Adventitious shoot regeneration of *M. lanceolata* starting with leaf explants (a & b) 4 weeks after incubation and (c & d) 8 weeks after incubation on selective medium. Control leaf discs did not show signs of callus formation and lost chlorophyll over time (a & c). Agroinfiltrated leaves and the transgenic shoot regeneration from infiltrated leaves (b & d). *Bar* = 1 cm.

PCR analysis

To determine whether the transgenic shoots carried NLS::GFP-containing T-DNA constructs, we analyzed eight regenerated shoots by PCR. A comparison was made between genomic DNA isolated from the transformed and non-transformed shoots. pK7FWGF2 plasmid was used as a positive control. Primers were designed for the amplification of the *npt*II kanamycin resistance gene. PCR reactions using DNA from non-transformed shoots produced two fragments but these did not correspond in size to what is predicted from the *npt*II sequence. The same fragments were also detected in PCR reactions with DNA from candidate transgenic shoots. However, the samples from candidate transgenic shoots generated a PCR fragment of about 400 bp that corresponds to the *npt*II amplification product for the selected primers (Fig. 6a). We therefore conclude that the *npt*II primers generated unspecific as well as *npt*II derived PCR products. To evaluate the presence of GFP sequences in transgenic shoots, PCR reactions using GFP-specific primers were conducted. All the candidate transgenic shoots generated a PCR fragment of 800 bp which corresponds

to the predicted *GFP* PCR product (Fig. 6b). To determine whether extracellular *A. tumefaciens* proliferation would be the template source for GFP PCR amplification, we performed PCR analysis with primers that amplify the genomic *Agrobacterium vir*G and *vir*C genes. The analysis showed that the predicted PCR product is obtained with *Agrobacterium* DNA extracts as template, but not when plant extracts were used (Fig. 6c, d). We therefore conclude that there was no *Agrobacterium* contamination in the extract of transgenic plants.

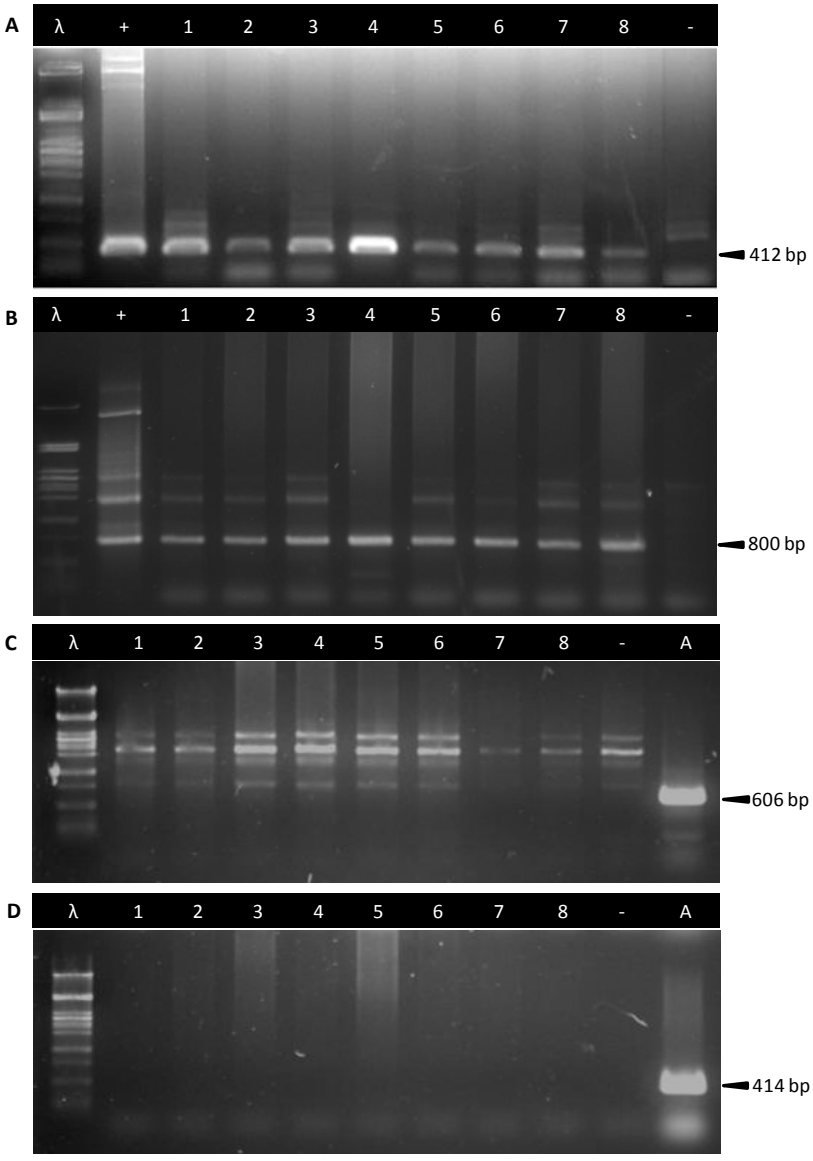


Fig. 6 PCR analysis of plants regenerated on kanamycin-containing medium. Genomic DNA was isolated from 8 individual transformation experiments. The PCR products of amplified 412 bp (a), 800 bp (b), 606 bp (c), and 414 bp(d) fragments corresponding to the *npt*II kanamycin resistance, *GFP*, *vir*G and *vir*C genes respectively. λ = Lambda DNA/PstI standard, + = positive control (plasmid pK7FWGF2), lane 1-8 = transformed plants, - = negative control (untransformed plant) and A = *A. tumefaciens.*

3. Discussion

Transient gene expression

It is known that some *Agrobacterium* strains are more virulent than others, depending on the target plant species. For example, it has been indicated that the C58 strain is most suitable for the transient expression assay in lettuce, tobacco and Arabidopsis (Wroblewski et al., 2005). Previous reports suggest that variations in transformation efficiency are caused by several factors including differences in ability of the bacterial cells to attach to plant cells, and differences in *vir* region and the chromosomal background (Nam et al., 1997; Chen et al., 2010). Because these factors are not likely influenced by different incubation conditions all subsequent experiments were performed using the LBA4404 strain. Much less is known about the host components which also affect the recognition process, the transfer of T-DNA, and the integration of T-DNA (Kim et al., 2009). To minimize variations in transformation efficiencies, we used *in vitro* clonally propagated shoot material.

The density of *Agrobacterium* suspension had a significant effect on GFP transient expression. The results indicate that the GFP expression occurred at OD_{600} values between 0.1 and 1.6 with maximum expression level at bacterial density of OD 1.0. This is similar to findings in grapevine (Santos-Rosa et al., 2008) and Arabidopsis (Kim et al., 2009) where densities as low as OD 0.1 induced at least a weak expression. In contrast, the transient gene expression in rose petals was only observed for bacterial densities greater than 0.5 (Yasmin and Debener, 2010). Necrosis and withering have been reported in tobacco and tomato to depend on the bacterial density (Wroblewski et al., 2005). In our study, however, necrosis and withering of *M. lanceolata* leaves did not occur in any of the tested conditions.

Acetosyringone is already known for its ability to activate virulence genes of *Agrobacterium* necessary to transfer T-DNA (Gelvin, 2003). There are several reports that show acetosyringone significantly improves the transient expression of foreign genes (Kapila et al., 1997; Ozawa and Takaiwa, 2010; Subramanyam et al., 2011). In contrast to these studies, the addition of acetosyringone did not cause transient transformation in lettuce, tomato, *Arabidospis* (Wroblewski et al., 2005), and rose petals (Yasmin and Debener, 2010). The presence of acetosyringone in agroinfiltration buffer was essential for maximum transient expression in *M. lanceolata*. Our results showed that the addition of 100 µM of

acetosyringone increased the transient expression by approximately threefold compared to those without additional acetosyringone.

RNA silencing or PTGS has also been considered to play a major role in the post-introduction gene expression decline in transient expression (Voinnet et al., 2003; Dhillon et al., 2009). Therefore, we evaluated one of the suppressors of plant gene silencing, *p19* to combat the PTGS response from *M. lanceolata*. *p19* is a suppressor isolated from *Tomato bushy stunt virus* (TBSV) and inhibits the formation of the initiator RNA-inducing silencing complex (RISC) either by preventing the unwinding of duplex siRNAs or by sequestering the duplex siRNAs (Lakatos et al., 2004). However, we consistently have approximately a threefold decrease in frequency of transient expression when *p19* was co-transfected. Wroblewski and co-workers reported that the co-introduction of viral silencing suppressor clearly resulted in stronger transient gene expression in *Nicotiana benthamiana* (Wroblewski et al., 2005). However, none of tested silencing suppressors had an observable effect on transient GFP or GUS expression in lettuce and Arabidopsis (Wroblewski et al., 2005). Therefore, the ability of silencing suppressor like *p19* to prevent PTGS of transiently expressed genes appears to be specific on the plant genotype.

Stable transformation

The optimized transient *A. tumefaciens*-mediated transformation technique for *M. lanceolata* involves a 5 days co-cultivation with the *Agrobacterium* strain LBA4404 and OD_{600} of 1.0 supplemented with 100 µL acetosyringone. In the absence of successful transformation with submerged leaf tissue, we dissected the transfected leaf material and used an established *in vitro* regeneration protocol of *Maesa* (Faizal et al., 2011) for the regeneration of transgenic tissues of *M. lanceolata*. Shoot regeneration was stimulated in the presence of 250-500 mg/L cefotaxime. The same effect was reported in *Eucalyptus tereticornis* where the addition of 500 mg/L cefotaxime increased the number of shoot regeneration (Aggarwal et al., 2010). The increasing number of regenerated shoots could be due to changes in the endogenous levels of plant growth regulators (PGRs). It was reported that the regulatory activities of cefotaxime could be due to its interference with the metabolism of PGRs (Plus et al., 1993). In our study, however, the agroinfiltrated leaves produced the same average number of shoots as the control or about 3 shoots per leaf

explant. This showed that the number of regenerated shoots is limited by kanamycin, which only allowed the transformed cells to regenerate.

In the current study, we compared immersion of *M. lanceolata* leaf explants in *Agrobacterium* suspensions with agroinfiltrated material. Leaf discs immersed with *Agrobacterium* suspension did not show shoot regeneration and lost chlorophyll over time on SIM supplemented with 100 mg/L kanamycin. The same result was obtained on control/non-infiltrated leaves. In contrast, a number of plant cells could be regenerated and stably transformed by agroinfiltration. Similar to our findings, using agroinfiltration in the creation of marker-free tobacco plants was also more successful than using the immersion technique (Kopertekh and Schiemann, 2005; Jia et al., 2007). We speculate that agroinfiltration has an advantage over immersion because in agroinfiltrated tissue more plant cells are accessible to *Agrobacterium* adherence and interaction. It will be of interest to see if the technique also promotes transformation effectiveness in other plant species.

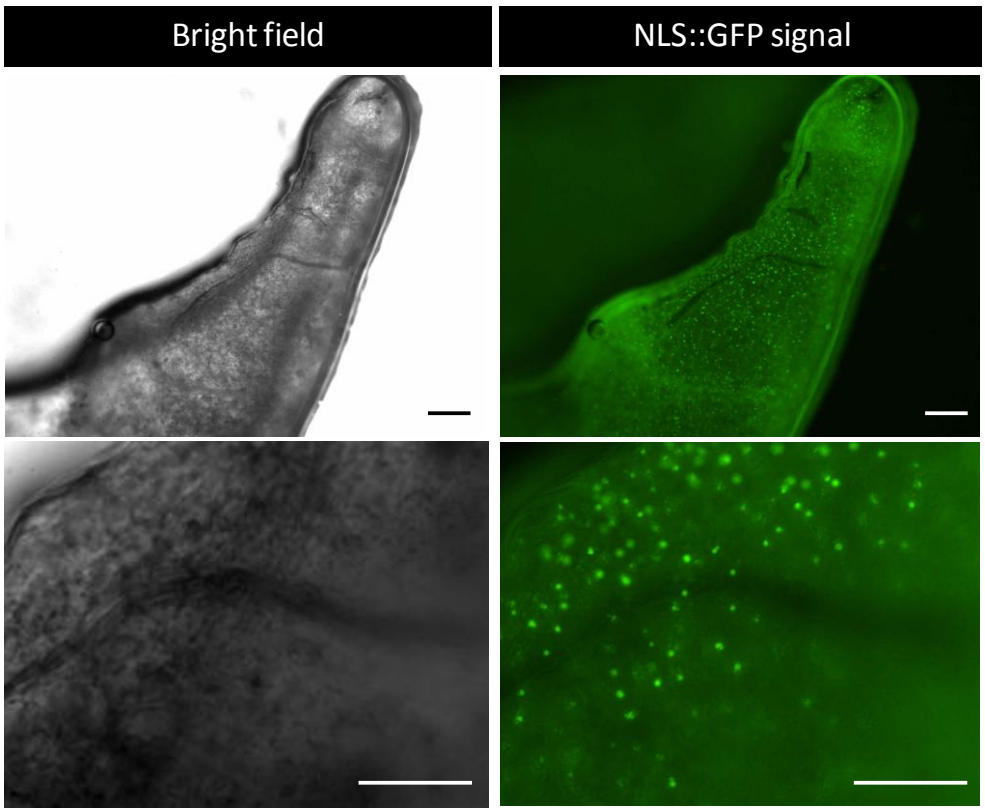


Fig. 7 Microscopy images showing the nuclear-targeted GFP of the *in vitro* regenerated shoots. *Bar* = 100 µm.

The stability of transformed *M. lanceolata* was easily visualized because the majority of cells showed nuclear-targeted GFP expression (Fig. 7) and this GFP expression was stable even in plants grown in the greenhouse (data not shown). Further confirmation of T-DNA integration was obtained by PCR analysis for eight independent shoot lines. Ongoing transformation experiments have shown that regenerated shoots are invariably transgenic (data not shown). The *vir*G and *vir*C PCR analysis supports the absence of *Agrobacterium* contamination affirming that the NLS::GFP-expressing shoots are genuine stable transformants.

In conclusion, the protocol reported here for *Agrobacterium*-mediated transformation of *M. lanceolata* is very efficient for high frequency transient gene expression of leaf tissues. In this study, we also proved that agroinfiltration can be used as an alternative approach to regenerate stably transformed plants. The stably transformed plants could be obtained in 3-4 months. On this basis, now we have a technique for engineering saponin biosynthesis from *M. lanceolata*. This technique can also be applied to other plant species that are permissive for agroinfiltration.

4. Materials and methods

A. tumefaciens strain and expression vector

The pK7FWGF2 binary vector (Karimi et al., 2002) carrying an NLS::GFP (*Nuclear Localization Signal, Green Fluorescence Protein*) construct driven by 35S promoter was introduced to competent *A. tumefaciens* strain LBA4404 by heat shock transformation. The binary vector also contains an *npt*II kanamycin resistance gene for plant selection. The 35S::*p19* was made by inserting the PCR-amplified fragment of the *p19* into SmaI-linearised pBin61 (Voinnet et al., 2003).

Three days prior to infiltration, a single colony of *Agrobacterium* was started as preculture for 48 h on YEB medium (beef extract 5 g/L, yeast extract 1 g/L, peptone 5 g/L, sucrose 5 g/L and 2 mM $MgSO_4.7H_2O$) supplemented with rifampicin 50 mg/L and spectinomycin 50 mg/L. Small aliquots of the preculture were transferred to fresh YEB medium with the same antibiotics and grown overnight with density of about OD_{600} = 1.5. The cultures were then centrifuged and the collected cells resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 100 µM acetosyringone) and OD_{600} was

adjusted to 1.0. The suspension was then incubated for 2 h at room temperature prior to infiltration. The virulence of the *Agrobacterium* was modulated by supplementing the bacterial culture medium with 0, 50, 100, 150 and 200 µM acetosyringone.

Agroinfiltration and immersion of *M. lanceolata* leaves

M. lanceolata was routinely acclimatized in the greenhouse from in vitro culture, 3 weeks prior to agroinfiltration. Agroinfiltration was performed as previously described (Voinnet et al., 2003), with some modifications. The 2nd – 5th young leaves counting from the apex were used for infiltration. Approximately 0.5 mL of the *Agrobacterium* cell suspension was injected at the abaxial side using a 5 mL syringe. Two or three injections were sufficient to infiltrate the cell suspension throughout each leaf. After infiltration, the plants were placed back under the same growth conditions. Co-infiltration with *p19* was done by mixing the bacterial cultures with final OD_{600} adjusted to 1.0.

For the immersion technique, leaf discs were immersed for 10 min in an *Agrobacterium* culture of OD_{600} 1.0 resuspended in infiltration medium. Treated leaf discs were blotted dry on sterile filter paper and placed adaxial side down on shoot induction medium without antibiotics. After 5 days of co-cultivation, leaf discs were transferred to shoot induction medium (SIM) containing MS medium (Murashige and Skoog, 1962) supplemented with 22.2 µM TDZ, 1.35 µM NAA, 3 % sucrose, and 0.8 % agar to induce adventitious shoot formation. 500 mg/L cefotaxime and 100 mg/L kanamycin were also added to SIM to select the transgenic shoots.

Evaluation of transient gene expression

Pieces of leaf were randomly cut from injected areas and mounted on slides for microscopic observation. To track the GFP expression, an inverted Olympus IX81 microscope with CellM™ software (Olympus) was used equipped with an XM-10 (Olympus) camera. The number of fluorescence nuclei in each image (1373 x 1038 pixel size) was calculated using ImageJ software (Rasband, 1997-2009). Only those fluorescence nuclei larger than 10 pixels were used for quantification.

After evaluation of GFP expression, agroinfiltrated leaves of *M. lanceolata* were cut and fixated with ethanol:acetic acid buffer (3:1) overnight. Subsequently, leaves were washed with water to remove the remaining fixative solution. For nuclei staining, leaves

were incubated with a 2 ng/µL DAPI (4',6' diamino-2-phenylindole) solution for 30 min followed by washing with water to reduce background interference and mounted on slides for microscopic observation. Frequency of transient transformation was scored by comparing the number of GFP-nuclei with the number of DAPI-stained nuclei, multiplied by 100 % [(n GFP/n DAPI) x 100].

Stable transformation from transient gene assay

Effect of cefotaxime

Cefotaxime was used to prevent the over growth of *Agrobacterium*. To determine the optimum cefotaxime concentration for maximum shoot regeneration, we incubated leaf discs on shoot induction medium containing different concentrations of cefotaxime (0, 125, 250 and 500 mg/L). The cultures were incubated with same growth conditions as mentioned above.

In vitro establishment of infiltrated leaves

For stable transformation, the infiltrated leaves were sterilized for 30 min with 10 % (v/v) solution of Haz-tab solution and a drop of Dreft^{TM} detergent (Procter and Gamble, UK) and rinsed three times with distilled water. Sterilized explants were placed on shoot induction medium supplemented with 500 mg/L cefotaxime and 100 mg/L kanamycin to select the transgenic plants. Multiple shoots were induced through adventitious shoot regeneration as previously described (Faizal et al., 2011).

PCR analysis

Leaf samples of approximately 100 mg of *in vitro* regenerated shoots were collected for genomic DNA isolation. The collected samples were frozen immediately in liquid nitrogen and genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen). For PCR analysis, primers were designed for the amplification of the *npt*II kanamycin resistance gene and *GFP* gene. The 412 bp fragment of *npt*II was amplified using oligo-nucleotide primers of 5'-GATGTTTCGCTTGGTGGTC-3' and 5'-GAACAAGATGGATTGCACGC-3'. The 800 bp of *GFP* was amplified using oligo-nucleotide primers of 5'-TAGTCGACCTGCAGGCGGC-3' and 5'-TTTCTCGAGTTACTTGTACAGCTCGTCCATGCC-3'. *vir*G primers (5'-GCCGGGGCGAGACCATAGG-

3' and 5'-CGCACGCGCAAGGCAACC-3'), which amplify the 606 bp fragment of *vir*G gene (Schaart et al., 2004) and *vir*C primers (5'-GGCGGGCGCGCCGAAAGGAAAACCT-3' and 5'-AAGAACGCGGAATGTTGCATCTTAC-3'), which amplify the 414 bp of *vir*C gene (Suzaki et al., 2004) of *A. tumefaciens* were used to determine the presence of *A. tumefaciens*. The following cycling conditions were tested: 95 ºC for 2 min; 30 cycles at 95 ºC during 30 s, 54 ºC during 30 s, 72 ºC during 30 s for *npt*II and *vir*C or 1 min for *GFP* and *vir*G; followed by a final incubation at 72 ºC for 5 min. The amplified products were separated on 0.8 % agarose gel electrophoresis and visualized with ethidium bromide.

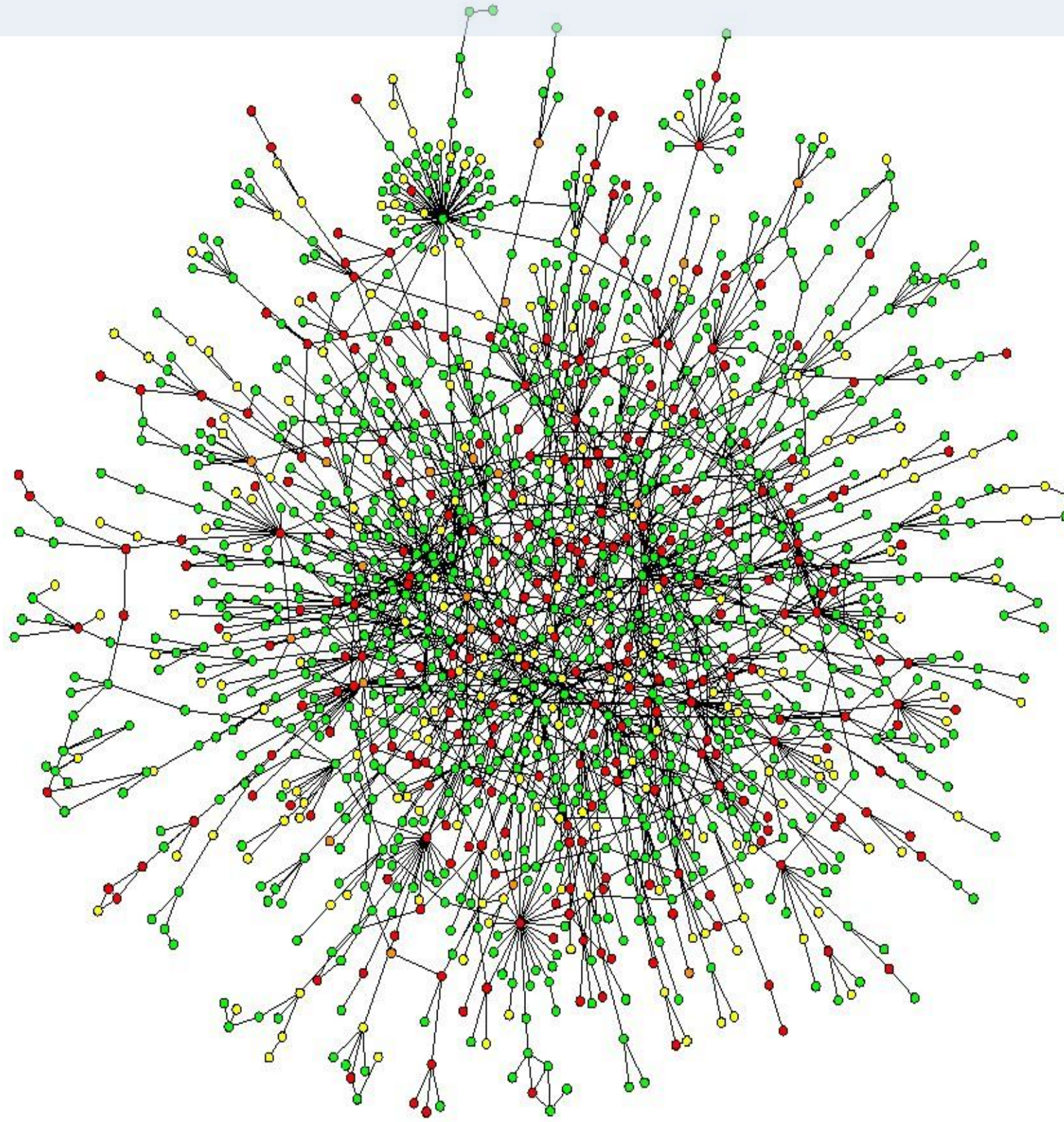
Statistical analysis

Data were analyzed using one-way ANOVA and the comparisons of the mean GFP expression was contrasted using Duncan's multiple range test. All statistical analyses were performed at the level of *P*-value less than 0.05 using SPSS 18.0 (SPSS Inc. USA)

Acknowledgments

The authors acknowledge David Baulcombe from Sainsbury Laboratory, Norwich, United Kingdom for providing the *p19* construct. We also thank the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia for providing financial support for A.F.

6 Regulation of β-amyrin synthase in triterpene saponin production of *Maesa lanceolata*



Cover art: *The yeast protein interaction network* | www.bordalierinstitute.com/target1.html

6

Regulation of β-amyrin synthase in triterpene saponin production of *Maesa lanceolata*

Ahmad Faizal*, Jacob Pollier,
Alain Goossens, and Danny Geelen

Author contributions:
* *Agrobacterium*-mediated transformation, generating RT-PCR, saponin extraction, TLC analysis, data analysis and writing the chapter.

Abstract

Maesasaponins are an oleanane type of saponins produced by *Maesa lanceolata*, which involve the activity of oxidosqualene cyclase (OSC), specifically β-amyrin synthase. In this study, the full length cDNA of β-amyrin synthase, designated *Ml-b*AS was isolated from *M. lanceolata*. The *Ml-b*AS open reading frame consists of 2283 bp encoding a protein of 760 amino acid residues. This amino acid sequence has QW and DCTAE motifs, which are highly conserved among the known triterpene synthases. In addition, the comparative and phylogenetic analysis showed that *Ml-b*AS was closely related to other plant OSCs. RT-PCR analysis indicates that *Ml-b*AS is expressed in all organs, but accumulates strongly in the leaf. RNA interference of *Ml-b*AS in transgenic *M. lanceolata* sufficiently suppressed *Ml-b*AS transcription. Concomitantly, this resulted in reduction of maesasaponin production. Interestingly, overexpressing *Ml-b*AS resulted in differential accumulation of saponin. In some cases the overexpression of *Ml-b*AS gene lead to enhanced saponin accumulation, but in other cases also leads to co-suppression resulting in a reduction of saponin. These findings indicate that the expression of *Ml-b*AS in *M. lanceolata* directly correlates with saponin production.

1. Introduction

Triterpenes are one of the largest groups of plant metabolites, with a diverse range of properties and nearly 200 distinct skeletons so far identified (Xu et al., 2004). The most studied triterpenes in higher plants are those from the oleanane type (β-amyrin), followed by ursane (α-amyrin), lupane and dammarane types (Shibuya et al., 2009; Woldemariam et al., 2011). All these triterpenes are synthesized in plants, via the cyclization of the common precursor 2,3-oxidosqualene. This conversion is catalyzed by specific oxidosqualene cyclases (OSCs) including β-amyrin synthase. The genes encoding β-amyrin synthase have been cloned and characterized from different plant species such as *Panax ginseng* (Kushiro et al., 1998), *Pisum sativum* (Arramon et al., 2002), *Glycyrrhiza glabra* (Hayashi et al., 2001), *Avena strigosa* (Haralampidis et al., 2001), *Medicago truncatula* and *Lotus japonicus* (Iturbe-Ormaetxe et al., 2003) and many other plants (Zhang et al., 2003; Kajikawa et al., 2005; Meesapyodsuk et al., 2007; Cammareri et al., 2008; Liu et al., 2009; Scholz et al., 2009; Woldemariam et al., 2011).

β-amyrin synthase (*b*AS) is one of the OSCs located at the branch point for sterol (primary metabolism) and triterpene saponin (secondary metabolism) biosynthesis. Therefore, knowledge about the regulation of β-amyrin synthase will be critical for understanding the regulation, mechanism and physiology of saponin secondary metabolism. Since the product of β-amyrin synthase is one of the key precursors of the triterpene saponin pathway, overexpression of β-amyrin synthase is an intelligible strategy to attain increased saponin production. This chapter describes our effort to modulate β-amyrin synthase activity in *Maesa lanceolata.*

Maesasaponins are of the oleanane type, which share a common skeleton consisting of β-amyrin. However, the detailed biosynthetic pathway has remained unresolved in *M. lanceolata* and β-amyrin synthase unidentified. In this study, we aim to isolate and misexpress *M. lanceolata* β-amyrin synthase (*Ml-b*AS). For this, we have transformed *M. lanceolata* plants with RNAi and overexpression vectors under the control of a constitutive 35S promoter. By applying these approaches, we obtained transgenic plants with differential *bAS* expression. The saponin production was analyzed through qualitative TLC analysis. The results show that *Ml-b*AS expression directly correlates with triterpene saponin production in *M. lanceolata*.

2. Results

cDNA cloning and phylogenetic analysis of *Ml-b*AS

During cDNA-AFLP transcript profiling of MeJA elicitated gene tags from *M. lanceolata* (executed in Department of Plant System Biology, PSB-VIB/Ghent University), we obtained a set of 36 gene tags, which were potentially involved in saponin biosynthesis pathway (Pollier et al., 2011). However, using cDNA-AFLP, none of these tags corresponded to β-amyrin synthase. Therefore, we amplified a gene tag corresponding to *M. lanceolata*-*b*AS using degenerate oligonucleotide primers based on known plant genes. The obtained sequence allowed generating a full-length open reading frames of *Ml-b*AS from a *M. lanceolata* cDNA library (Pollier et al., 2011).

The *Ml-b*AS open reading frame (ORF) consisted of 2,283 bp nucleotides encoding a 760 amino acid protein of about 87.5 kDa. The *Ml-b*AS amino acid sequence exhibits 77%, 75%, 74% and 74% identity with *b*AS from *P. ginseng* (O82146), *G. glabra* (Q9MB42), *M. truncatula* (XP003604121) and *Pisum sativum* (Q9LRH8) respectively. The *b*AS amino acid sequence contains QW and DCTAE motifs, which are highly conserved among the known triterpene synthases (Fig. 1). In addition to these motifs, the MWCYCR motif is also present, containing a tryptophan residue important for β-amyrin enzyme activity as has been reported in pea (Kushiro et al., 2000).

In order to better understand the relationship between OSC in different species, we constructed a phylogenetic tree using the different deduced amino acid sequences of OSCs from different plants (Fig. 2). Phylogenic analysis indicates that *Ml-b*AS shares similarities with other β-amyrins as well as with lupeol and cycloarthenol synthases supporting the hypothesis that OSCs of sterol and triterpene saponins may share a common ancestral origin (Haralampidis et al., 2002).

Expression of *Ml-bAS* mRNAs in different organs

To investigate *Ml-b*AS transcription, total RNA was isolated from leaves, stems and roots and subsequently examined by reverse transcription-PCR (RT-PCR). As shown in Fig. 3, *Ml-b*AS was expressed ubiquitously in various organs, but mRNA is more abundant in leaves compared to other organs. This result is in agreement with the high accumulation of

maesasaponin in leaves (chapter 3). Thus, the cloned *Ml-b*AS expression correlates with the site of maesasaponin biosynthesis.

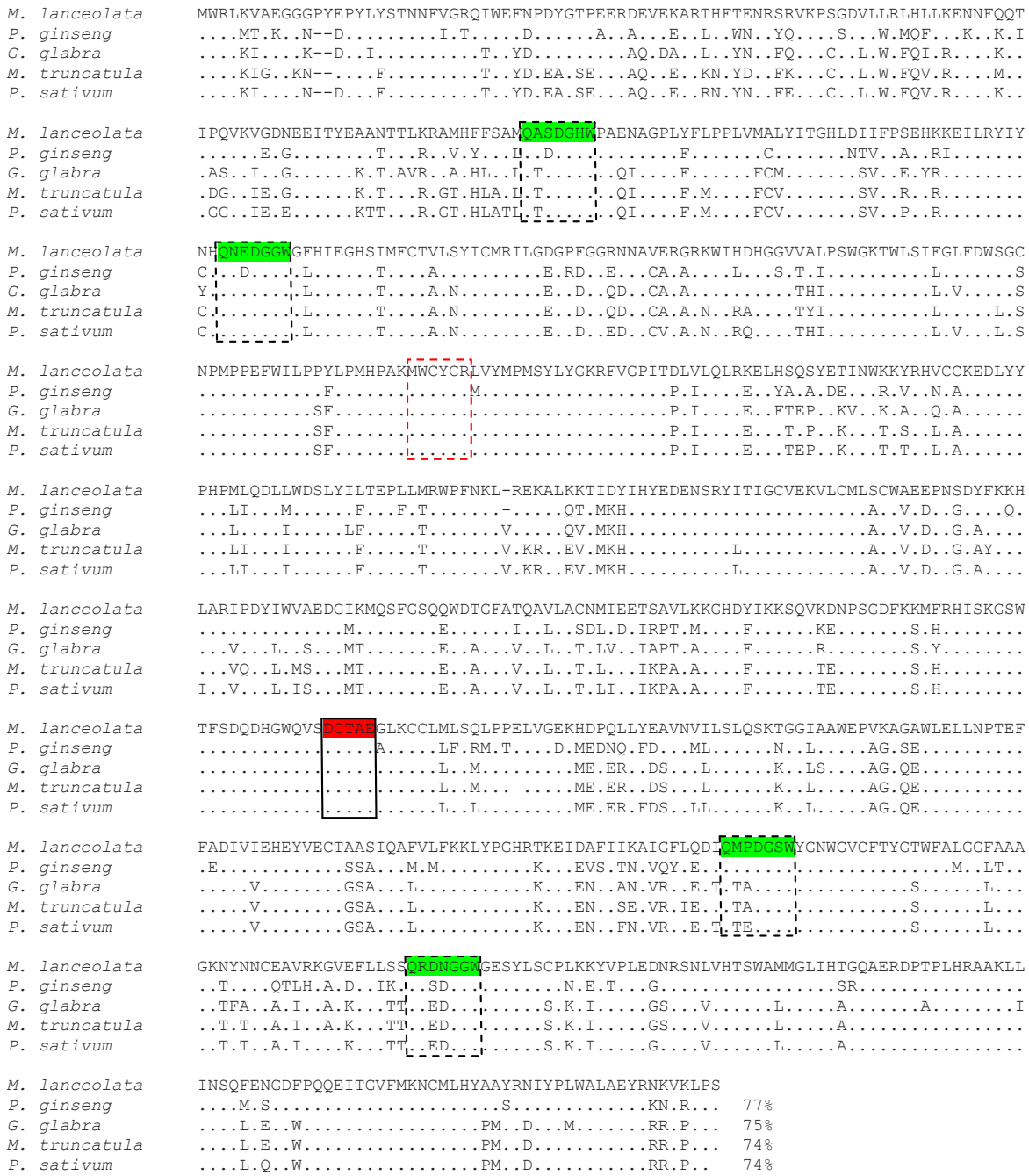


Fig. 1 Alignment of the deduced amino acid sequences of *M. lanceolata* and other β-amyrin synthases. The DCTAE motif is boxed, the QW and MWCYCR motifs are boxed by broken line. Dots indicate residues that are identical to those in the *Ml-b*AS sequence, and dashes indicate alignment gaps. The sequence identities between the *Ml-b*AS sequence and the other sequences are indicated at the end of each sequence. The GenBank accession numbers of the sequences are O82146 (*P. ginseng*), Q9MB42 (*G. glabra*), XP_003604121 (*M. truncatula*) and Q9LRH8 (*P. sativum*)

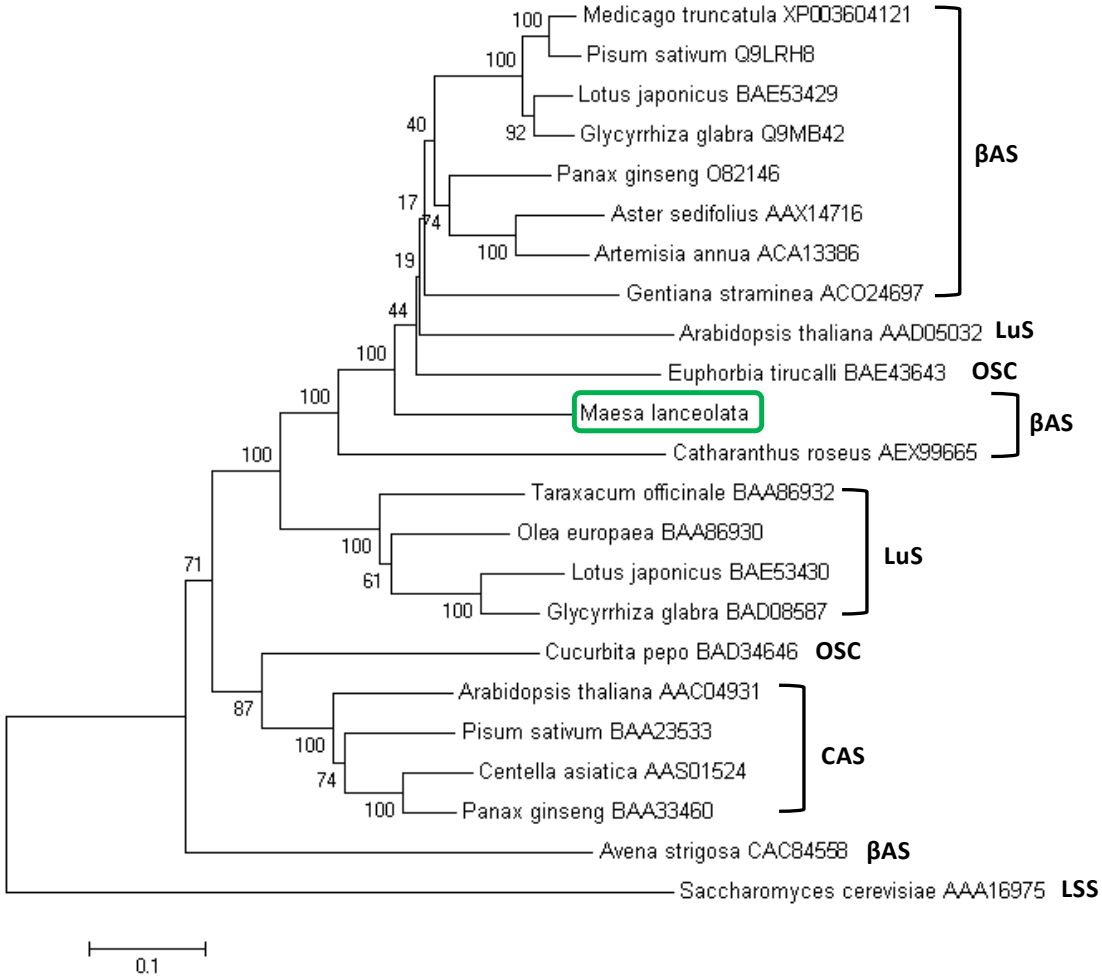


Fig. 2 Phylogenetic tree analysis of oxidosqualene cyclases (OSCs) from different organism constructed by the neighbor-joining method. The lanosterol synthase (LSS) of *Saccharomyces cerevisiae* was used as the outgroup. The accession numbers from GenBank are written after species name. Distances between each clone and group are calculated with Mega 4 program. βAS = β-amyrin synthase, LuS = lupeol synthase, CAS = cycloarthenol synthase

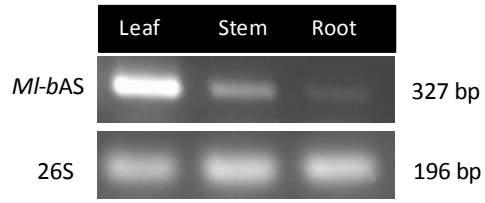


Fig. 3 The expression of *Ml-b*AS in different organs of *M. lanceolata*

Saponin production is suppressed by RNAi-mediated silencing of *Ml-b*AS

To functionally analyze *Ml-b*AS *in planta*, transgenic *M. lanceolata* plants constitutively expressing an *Ml-b*AS-RNAi were constructed using the destination vector pK7GWIWG2(II)

(Fig. 4a). Kanamycin-resistant RNAi adventitious shoots developed on the surfaces of leaf explants grown on selection media. The integration of *Ml-b*AS and neomycine phosphotransferase (*NPT* II) genes in kanamycin-resistant plantlets was confirmed by PCR (as previously shown in chapter 5). Such kanamycin-resistant lines were screened by RT-PCR which showed that the accumulation of *Ml-b*AS was variably reduced in independent RNAi lines (Fig. 4b). Furthermore, to have a clear correlation between *Ml-b*AS transcript level and triterpene saponin production, we performed a qualitative TLC at the same time, with the same samples for RT-PCR. TLC analysis revealed that maesasaponin content was reduced in RNAi lines with a corresponding reduction in *Ml-b*AS transcript level (Fig. 4c). This result provides direct evidence that *Ml-b*AS plays a critical role in saponin biosynthesis in *M. lanceolata*.

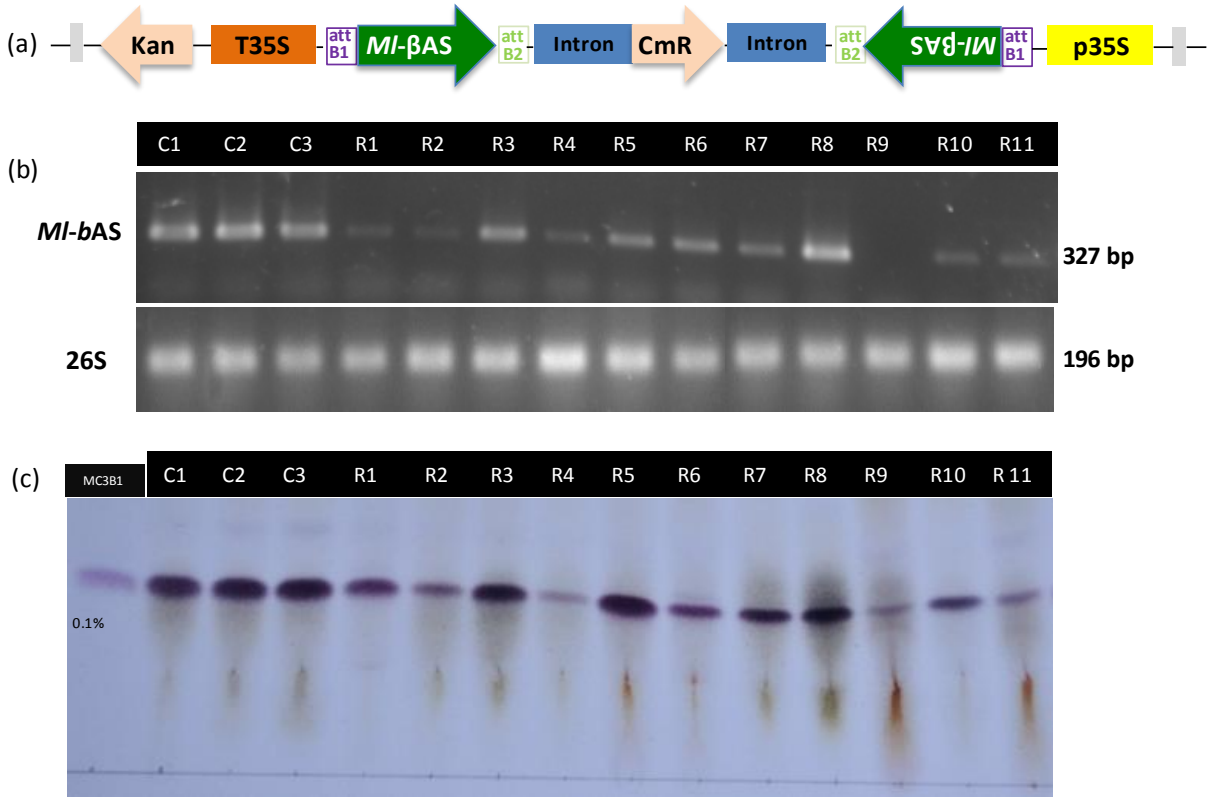


Fig. 4 Posttranscriptional silencing of *Ml-b*AS in *M. lanceolata*. (a) Schematic representation of pK7GWIWG2(II)-*Ml-b*AS-RNAi vector. (b) Expression of *Ml-b*AS in leaves of wild-type control and *Ml-b*AS-RNAi transgenic plants by RT-PCR analysis. (c) Saponin content in leaves of control plants and RNAi lines. L= DNA ladder (the size of its fragments is indicated on the right), C1-C3= control plants; R1-R11 = RNAi lines; MC3B1 = An HPLC purified maesasaponin mix (reference sample).

Differential expression of the *bAS* gene influences the accumulation of triterpene saponin in *M. lanceolata* overexpressing *Ml-b*AS

The accumulation of *Ml-b*AS *mRNA* was analyzed in leaves of transgenic plants by RT-PCR. Overexpression of the *Ml-b*AS gene in *M. lanceolata* caused differential accumulation of *Ml-b*AS mRNA in transgenic lines. The transcript of *Ml-b*AS was found to be highly accumulated in two lines: O2 and O5. Interestingly, co-suppression occurred in three other lines (O1, O3 and O4) and resulted in less mRNA accumulation (Fig. 5a). These results indicate that the overexpression of the *Ml-b*AS gene may lead to both elevated and reduced *Ml-b*AS transcript levels. The level of *Ml-b*AS transcript correlated with the production of saponin in *M. lanceolata*.

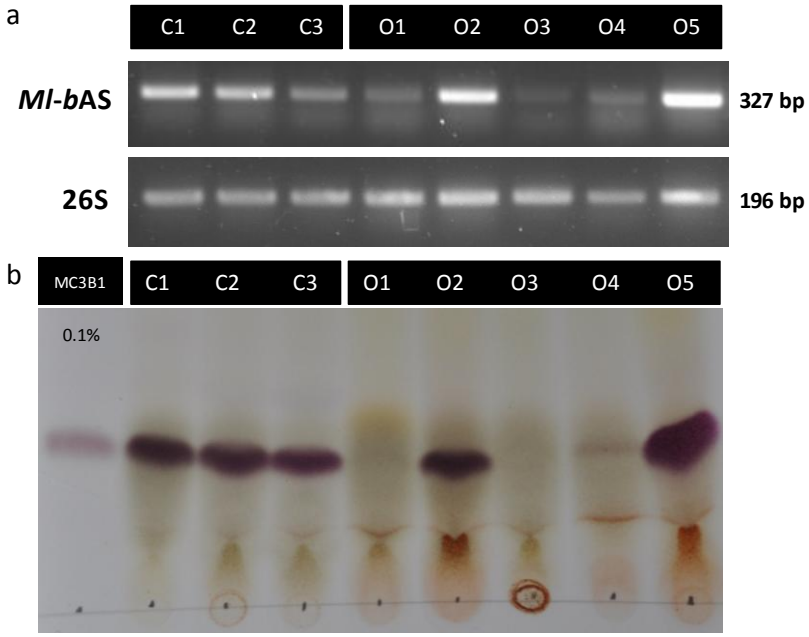


Fig. 5 Overexpression of *Ml-b*AS in *M. lanceolata*. (a) Expression of *Ml-b*AS in leaves of wild-type control and *Ml-b*AS overexpression plants by RT-PCR analysis. (b) Saponin content in leaves of control plants and overexpression lines. L= DNA ladder, C1-C3= control plants; O1-O5 = overexpression lines; MC3B1 = an HPLC purified maesasaponin mix (reference sample).

Highly expressed *bAS* in *M. lanceolata* affects plant development

Although no differences were observed in in vitro culture, the overexpression of *Ml-b*AS apparently affects the plant growth and development under greenhouse conditions (Fig 6). The plant shows a dwarf phenotype (Fig. 6b) and small leaves compared to the control (Fig 6c). We suggest that by overexpressing β-amyrin synthase, the pathway for sterol

production may be influenced resulting in a retarded plant growth. However, whether this was affected by sterols, still need to be determined.

Fig. 6 (a) Transgenic lines of *M. lanceolata* overexpressing *Ml-b*AS (O2, O3 and O4 look shorter than control because they have been pruned prior the image taking, also indicated by the growth of their axillary branching); (b) phenotypic comparison between control and O5 (saponin over producing line); (c) the difference on leaf size between control and O5. *Bar* = 5 cm

3. Discussion

In this study, a new β-amyrin synthase gene (*Ml-b*AS) with a function in triterpene biosynthesis was isolated from *M. lanceolata* through a similarity-based cloning strategy. This technique has been previously applied to isolate other β-amyrin synthase genes in different plant species (Kushiro et al., 1998; Iturbe-Ormaetxe et al., 2003; Kajikawa et al., 2005; Meesapyodsuk et al., 2007; Cammareri et al., 2008).

The deduced amino acid sequence of *Ml-b*AS protein exhibited QW and DCTAE motifs that are highly conserved among the known triterpene synthases. These motifs are also present in OSCs directing to sterol biosynthesis, demonstrating a close evolutionary relationship between sterol and triterpene biosynthesis as also reported in *P. ginseng* (Kushiro et al., 1998), *Olea europea* and *Taraxacum officinalis* (Shibuya et al., 1999). In addition, mutational analysis of other OSCs showed that the DCTAE motif is required for substrate binding whereas the QW motif is likely to be important for protein structure and stability, and for catalytic activity (Haralampidis et al., 2002).

Reverse transcription-PCR analysis of *Ml-b*AS revealed differential expression in different organs. In particular, *Ml-b*AS transcripts accumulated strongly in leaves followed by stems and roots. This expression pattern for *Ml-b*AS is in agreement with the phytochemical results which showed a higher content of triterpenoid saponins in *M. lanceolata* shoots compared to roots (see chapter 3 and Theunis et al. (2007)).

To gain some insight into the role of *Ml-b*AS in plants, we subsequently generated RNAi-mediated silencing lines by *Agrobacterium tumefaciens*-mediated transformation. As RNAi is a homology-dependent process, a specific gene or multiple members of a gene family can be silenced by targeting highly conserved sequence domains. Therefore, *Ml-b*AS-RNAi was constructed using a specific fragment of 388 bp nucleotides from 3' region including UTR. As such, we could generate RNAi lines with sufficient suppression of *Ml-b*AS transcript level.

A previous experiment with β-amyrin synthase-RNAi has been reported in seed of soybean (Takagi et al., 2011). Soybean seeds contain triterpenoid saponins that influence the seed quality. By using RNAi-mediated gene silencing targeted to β-amyrin synthase, six independent transgenic lines showed a stable reduction in seed saponin content corresponding to reduction of β-amyrin synthase transcript level. Although some transgenic

lines produced seeds almost devoid of saponins, no abnormality in their growth was shown. These results suggest that saponins are not required for seed development. Similarly, such deficiency did not appear to affect the plant growth and development of our RNAi-silenced lines. Therefore, we speculate that maesasaponins are not required for normal growth and development in *M. lanceolata*, although their production seems to be influenced by plant growth and development (chapter 3). A contradictory result was reported on the influence of β-amyrin synthase in seed germination of *Arabidopsis thaliana* (Tomilova et al., 2001). They obtained a T-DNA insertion mutant of *A. thaliana* corresponding to the C-terminal of *At1g78950* encoding a β-amyrin synthase which had shortened hypocotyls and no development of roots during germination. However, Shibuya et al. (2009) showed that the phenotype of this mutant cannot be simply due to the lack of β-amyrin, and/or its metabolites since β-amyrin was produced not only by the specific synthase At1g78950, but also by multiple OSCs, namely At1g78955, At1g78960 and At1g78970.

The production of triterpene saponins has been reported to be significantly up-regulated in *M. truncatula* expressing a *b*AS gene from *Aster sedifolius* (Confalonieri et al., 2009). In addition, a recent study has shown that pentacyclic β-amyrin, which naturally absent from Arabidopsis, accumulates in the intracuticular wax layer through ectopic expression of the triterpenoid synthase AtLUP4 in Arabidopsis leaves (Buschhaus and Jetter, 2012). Therefore, to enhance maesasaponin production we generated *b*AS overexpression lines of *M. lanceolata*. Overexpression of *Ml-b*AS in *M. lanceolata* resulted in differential *b*AS transcript accumulation. Of all overexpression lines, we produced two lines with increased transcript level with O5 showing a more dramatic increase. The other lines showed co-suppression. Co-suppression can arise when cis genes from the same plant are expressed to such high levels that it induces an RNA silencing mechanism, leading to reductions instead of increases in the production of target metabolites (Napoli et al., 1990; Depicker and Van Montagu, 1997). In co-suppression, double-stranded RNAs (dsRNAs) are synthesized by the plant-encoded RNA-dependent RNA polymerase6 (RDR6) on templates of sense transgene transcripts. A transgene that expresses dsRNAs can induce both transcriptional (TGS) and post-transcriptional gene silencing (PTGS) when the coding sequence and the promoter sequence are transcribed respectively (Sijen et al., 2001; Mourrain et al., 2007; Oka et al., 2010; Kasai et al., 2012).

Our studies show that *b*AS transcript levels were correlated with their saponin production with 2 co-suppressed lines showing depleted saponin production. Similar to RNAi-mediated lines, these lines also did not show any interference in plant growth and development while conversely the line that overproduces saponin corresponding to overexpressed *b*AS transcripts, has growth and developmental defects. As we know, *b*AS is one of the OSCs located at the branch point for sterol (primary metabolism) and triterpene saponin (secondary metabolism) biosynthesis (see Fig. 2 in chapter 1). Our data suggests that upregulation of *b*A*S* expression may disturb another pathway leading to sterol production, which is important in fundamental aspects of growth and development (Piironen et al., 2000; Lindsey et al., 2003).

In conclusion, this study shows that of *Ml-b*AS is likely a genuine β-amyrin synthase involved in the biosynthesis of maesasaponins. Whether *Ml-b*AS influences other pathways and/or specific products in triterpene saponin biosynthesis has yet to be determined. Additionally, it will be interesting to understand the regulation in maesasaponin biosynthesis by super transform the β-amyrin overexpressed line (O5) with other candidate genes that may act downstream in the saponin biosynthesis pathway.

4. Material and Methods

Full length open reading frame (FL-ORF) cloning of *Ml-b*AS

This work has been executed in Department of Plant System Biology, VIB-Ugent. Degenerate oligonucleotide primers were designed based on highly conserved amino acid regions of known plant *bAS* genes and used to amplify a fragment of the *Ml-b*AS gene. First a PCR was performed on *M. lanceolata* cDNA using the primers P1 and P2 (Table 1), corresponding to the amino acid motifs DGGWGLH and LKAARHLP, respectively. The amplicon was purified using the NucleoSpin Extract II kit (Macherey-Nagel) according to the manufacturer's instructions and used as a template for a nested PCR with the primers P3 and P4 (Table 1), corresponding to the amino acid motifs FLPMHPAKMW and EQAGAPEWA, respectively. The resulting PCR fragment was purified once again and cloned into the pGEM®-T easy vector (Promega) for sequencing. The obtained sequence was used to design the primers P5 and P6, which were used to generate a DNA probe for screening the FL-ORF of *Ml-b*AS in the *M.*

lanceolata cDNA library as reported (Pollier et al., 2011). The FL-ORF of *Ml-b*AS was PCR amplified for Gateway™ cloning in pDONR221 using the primer pairs P7 + P8 (Table 1).

Table 1. Primers used for *Ml-b*AS cloning

Primer	Sequence (5‘ to 3’)
P1	GAYGGNGGNTGGGGNYTNCA
P2	NARYTTNGCNGCYCTRTGNARNGG
P3	TTYYTNCCNATGCAYCCNGCNAARATGTGG
P4	TCYTGNGCNCCNGCNGGYTCCCANGC
P5	ATGTGGTGTTACTGTCGATTGG
P6	TAGCAAATCTTGCAGCATAGGA
P7	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCctcgagATGTGGAGGTTAAAGGTTCT
P8	GGGGACCACTTTGTACAAGAAAGCTGGGTCggtaccCTAAGACGGCAATTTGACCTTGTT

The sequences in lower case represent the restriction recognition site used for restriction enzyme mediated cloning.

Characterization and phylogenetic analysis of Ml-BAS.

The nucleotide and deduced amino acid sequences were analyzed and sequence comparison was conducted through a database search using BLAST tool (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was done by CLUSTAL W. The known OSCs amino acid sequences from different plants were downloaded from NCBI database and were aligned using CLUSTAL X2. A phylogenetic tree was created based on 500 bootstrap replications with the MEGA 4 program by neighbor-joining method.

Generation of *Ml-b*AS-RNAi silencing construct

Two primers, including Gateway adapters, were designed to amplify the region from 3’ region including UTR of *Ml-b*AS (388 nucleotides): forward primer, 5’-GGGGACAAGTTTGTACAAAAAAGCaggctcagtcctctcaagaaatacgtacc-3’ and reverse primer, 5’-GGGGACCACTTTGTACAAGAAAGCTgggtctctgtcagcagaaaagatccggc-3’ (the capital letters represent the adapters).

The amplified PCR product was cloned in the pDONR221 vector and transferred to the RNAi destination vector pK7GWIWG2(II) (in which NPT II gene confers kanamycin resistance). The construct was sequenced and subsequently transformed into *Agrobacterium tumefaciens* LBA4404, using standard molecular biology techniques.

Generation of Ml-BAS overexpression construct

The full length open reading frame (FL-ORF) of *Ml-b*AS that has been cloned into the overexpression Gateway™ vector pK7WG2D was subsequently transformed into *A. tumefaciens* LBA4404.

Generation of transgenic plants

The *A. tumefaciens*-mediated transformation for both RNAi and overexpression construct was performed by agroinfiltration technique previously developed for *M. lanceolata* leaves (chapter 5).

RT-PCR

Leaves of *M. lanceolata* plants grown for 3 months in greenhouse conditions were harvested, frozen and ground in liquid nitrogen. Half of the sample was processed for reverse transcription PCR (RT-PCR) and the other half was prepared for saponin extraction. Total RNA was extracted with Concert™ Plant RNA Reagent (Invitrogen), and cDNA was prepared with SuperScript™ II Reverse Trancriptase (Invitrogen).

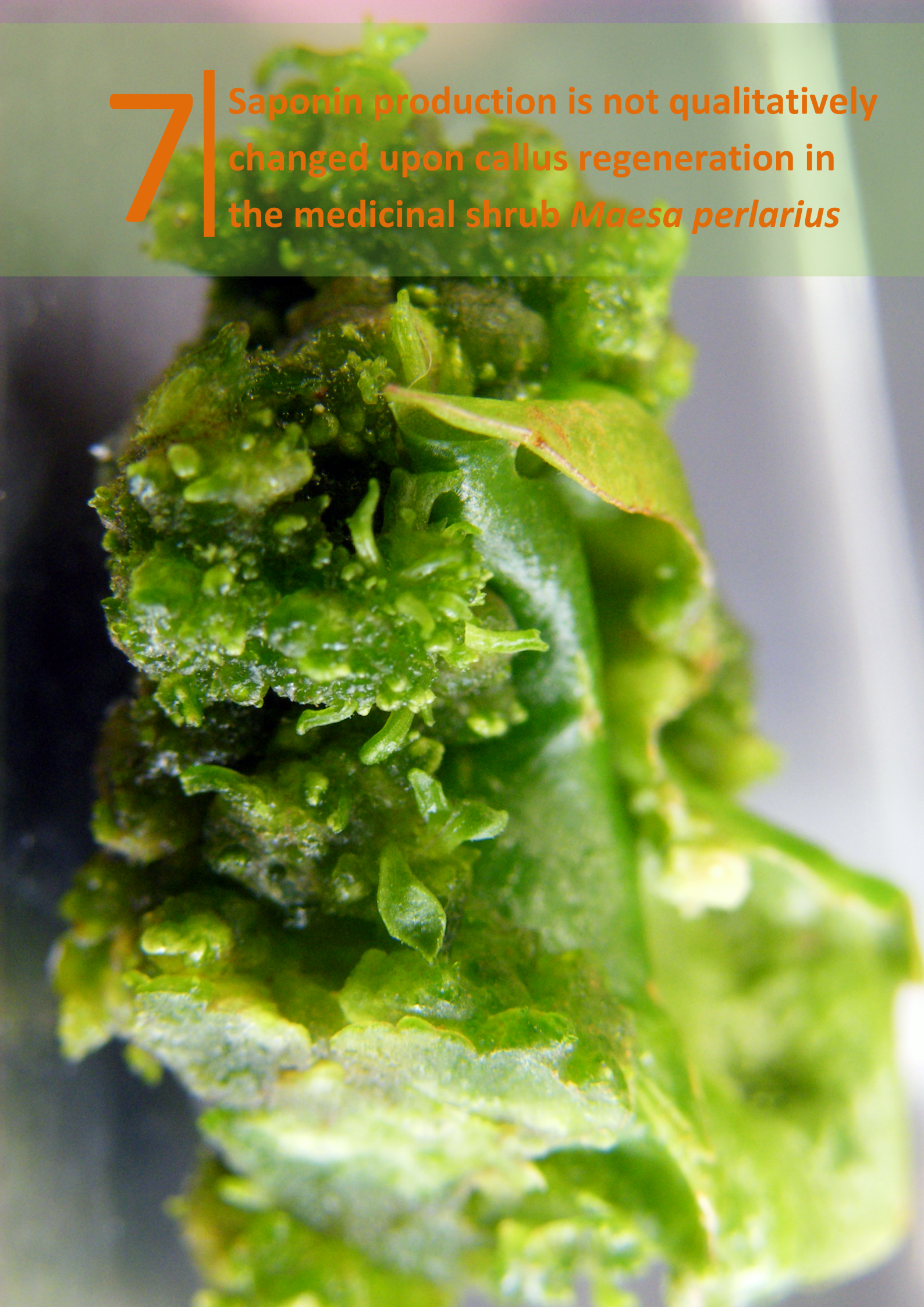
Saponin extraction and detection by TLC

Metabolite extraction and a qualitative TLC analysis for saponin detection are described in chapter 3.

Acknowledgement

We thank the Directorate General of Higher Education, Ministry of Education and Culture, Republic of Indonesia and the BOF (special research fund No. 01WI0111, Ghent University) for A.F. PhD fellowship.

7 | Saponin production is not qualitatively changed upon callus regeneration in the medicinal shrub *Maesa perlarius*



Cover art: Adventitious shoot regeneration of *Maesa perlarius*

7

Saponin production is not qualitatively changed upon callus regeneration in the medicinal shrub *Maesa perlarius*

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Published in:

Ahmad Faizal, Kenn Foubert, Ellen Lambert, Nico De Storme, Magda Claeys, Sandra Apers and Danny Geelen. **Saponin production is not qualitatively changed upon callus regeneration in the medicinal shrub *Maesa perlarius***. *Plant Growth Regulation* (2013), doi:10.1007/s10725-012-9776-1

* Author contributions: Plant maintenance, flow cytometry, morphological analysis, metabolite extraction, statistical analysis, and writing the chapter.

Abstract

Maesa perlarius is a medicinal plant that produces maesabalides, which possess selective and strong anti-leishmania activity. In this study, *M. perlarius* plants were regenerated from leaf-derived calli. Shoots were induced in Murashige and Skoog medium in the presence of thidiazuron (TDZ) in combination with α-naphthalene acetic acid (NAA). In contrast to seed-derived plants, callus-derived regenerants were tetraploid showing typical characteristics of higher ploidy phenotypes. We assessed the impact of indirect plant regeneration and associated increase in ploidy on the production of saponin by means of LC-MS analysis. Tetraploid *M. perlarius* produce a saponin profile, which was not significantly different from seed grown plants. Based on this study, we concluded that saponin production in *M. perlarius* is not qualitatively changed by a genome-doubling event.

1. Introduction

Maesa perlarius is a member of the Primulaceae and is a small shrub growing in the tropic of Asia. This plant is mainly found in Thailand, Vietnam and China and has been referred to traditional medicine and ancient practices (Wiart, 2006). Recent studies have shown that saponins produced from this *Maesa* species exhibit strong anti-leishmanial activity with low cytotoxicity (Foubert et al., 2009; Vermeersch et al., 2009).

Hence, it is important for pharmaceutical industries, prompting researchers to investigate the potential value of in vitro cultures for producing saponins under controlled condition. Plant tissue culture has become a feasible alternative to improve the efficiency of propagation of medicinal plants as well as to facilitate in vitro experiments such as genetic transformation, protoplast fusion and investigation of secondary metabolites production. However, tissue culture processes that involve an undifferentiated callus phase may cause a general disruption of cellular control, leading to genetic instability including polyploidy, aneuploidy, mutation, insertion of transposons and other numerous genomic changes present in tissue culture regenerants (Phillips et al., 1994; Lopez et al., 2010; Smulders and de Klerk, 2011).

Variation in ploidy level has been reported to have an impact on the yield of the secondary metabolite from in vitro grown cultures. For example, tetraploid hairy root cultures of *Artemisia* produced 6 times the artemisinin levels of diploid genotypes (De Jesus-Gonzalez and Weathers, 2003) and tetraploid hairy root cultures of *Hyoscyamus muticus* produced 2 times more of scopolamine than its diploid counterparts (Dehghan et al., 2012). A similar finding was reported for tetraploid *Catharanthus roseus* that produced more terpenoid indole alkaloids compared to diploid plants (Xing et al., 2011). In *Solanum commersonii,* the content of minor glycoalkaloids (solanidenediol triose, solanidadienol lycotetraose, and solanidenol lycotetraose) was higher in tetraploids, while the content of major glycoalkaloids (dehydrodemissine and dehydrocommersonine) was significantly higher in diploid genotypes (Caruso et al., 2011). Furthermore, induction of polyploidization may also alter the secondary metabolite of a plant in qualitative manner which is itself of potential value for qualitative improvement through ploidy manipulation (Levy, 1976; Lavania, 2005).

To evaluate the production of saponins from callus-derived plants of *M. perlarius*, we produced callus and induced shoot regeneration from callus and investigated ploidy variation and saponin production. The results show that saponin production is independent from a change in ploidy.

2. Results

Callus induction

For callus induction, in vitro leaves of *M. perlarius* were placed on solid MS medium supplemented with different concentrations and combinations of auxins and cytokinins, like benzyladenine (BA), kinetin (kin), α-naphthalene acetic acid (NAA) and 2**,**4-dichlorophenoxy acetic acid (2,4-D). Tested hormone combinations for callus induction on leaves of *M. perlarius* are represented in Table 1.

Table 1 Effect of plant growth regulators (PGRs) on the induction of callus on the leaf material of *M. perlarius*.

PGRs (µM)				Callusing	Morphology
NAA	BA	2,4-D	Kin		
-	-	-	-	no	
2.7	-	-	-	no	
-	4.4	-	-	no	
-	-	5.0	-	**yes**	**OS**
-	-	-	4.6	no	
2.7	4.4	-	-	no	
2.7	-	5.0	-	**yes**	**WS**
2.7	-	-	4.6	no	
-	4.4	5.0	-	**yes**	**OG**
-	4.4	-	4.6	no	
-	-	5.0	4.6	**yes**	**WS**

Callus formation was evaluated after 6 weeks. Experiments were performed in triplicate and callusing was always observed in each replicate. **O** = orange, **W** = white, **G** = granular, **S** = smooth.

Six weeks after induction, calli were observed and qualitatively analyzed (Fig. 1a). Leaf explants showed no response in the absence of growth regulators. Results also indicate that the auxin 2,4-D is necessary for callus induction. Callus was observed when 2,4-D was applied alone or in combination with NAA, BA or kin. Callus of *M. perlarius* was often very smooth and had a cream white to yellowish orange color.

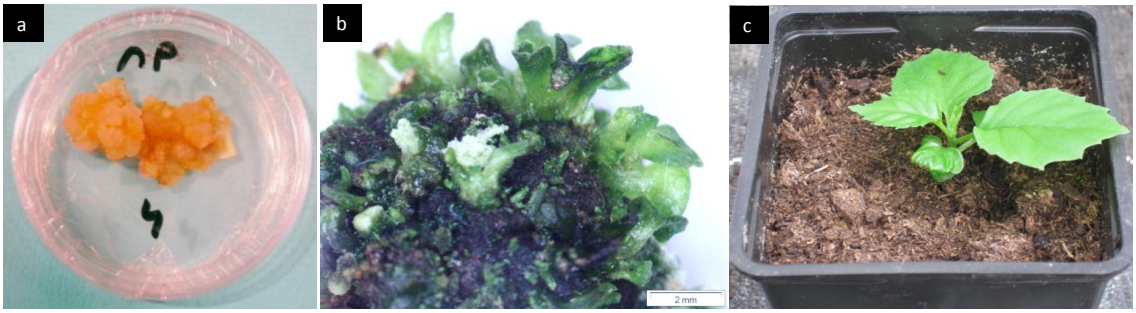

Fig. 1 Callus induction (a), callus regeneration (b) and acclimatization (c) of *M. perlarius.*

Callus regeneration

For this regeneration experiment, 1 year old calli of *M. perlarius* were placed on solid medium with 15 different BA and kin concentration and incubated in a growth room with 16/8 h light/dark condition and 25 °C. Six weeks after incubation, the calli had grown but did not yet show signs of shoot formation. In parallel with these tests, experiments were performed to induce adventitious shoots on *Maesa* leaf material and it was found that thidiazuron (TDZ) in combination with NAA gave better results than BA or kin. Therefore, calli on regeneration medium were split in two; half of the callus was placed on fresh medium with the same concentration of BA and/or kin and the other half of the callus was placed on medium with 4.5 µM TDZ and 0.05 µM NAA (Table 2).

In contrast to BA or kin, 4.5 µM TDZ combined with 0.05 µM NAA induced shoots on *M. perlarius* callus (Table 2). The formation of shoots was macroscopically visible after 17 weeks of incubation (Fig. 1b). *M. perlarius* calli produced 0.4 shoots per callus. We isolated 15 independent plantlets, which were subsequently elongated and spontaneously developed roots in basal MS media without phytohormones. Regenerated and rooted shoots were acclimatized with 100% survival rate (Fig. 1c).

Table 2 Shoot induction on callus of *M. perlarius.* Calli were first incubated on medium with BA and kin in different concentrations. After 6 weeks, half of the calli were placed on medium with 4.5 µM TDZ and 0.05 µM NAA. First shoots were observed 17 weeks after the beginning of the experiment.

PGRs (µM)				No. shoot / callus ± SEM	Roots
BA	Kin	TDZ	NAA		
-	-	-	-	0.0[a]	No
0.4	-	-	-	0.0[a]	No
2.2	-	-	-	0.0[a]	No
4.4	-	-	-	0.0[a]	No
-	0.5	-	-	0.0[a]	No
-	2.3	-	-	0.0[a]	No
-	4.6	-	-	0.0[a]	No
0.4	0.5	-	-	0.0[a]	No
0.4	2.3	-	-	0.0[a]	No
0.4	4.6	-	-	0.0[a]	No
2.2	0.5	-	-	0.0[a]	No
2.2	2.3	-	-	0.0[a]	No
2.2	4.6	-	-	0.0[a]	No
4.4	0.5	-	-	0.0[a]	No
4.4	2.3	-	-	0.0[a]	No
4.4	4.6	-	-	0.0[a]	No
-	-	4.5	0.05	0.4 ± 0.2[a]	Yes

The experiment was performed in triplicate with 9 repeats per replicate. Different letters indicate significant differences ($P < 0.05$) according to Duncan test.

Ploidy analysis

To investigate the *M. perlarius* callus-derived plants genetically, we first analyzed their ploidy using flow cytometry. A clear shift in ploidy was observed for the fluorescence peak of all *M. perlarius* regenerants compared to control plants (Fig. 2). Relative DNA content of controls and regenerants is presented in Table 3. The nuclear DNA content of all regenerated plants was 2 times that of the controls, indicating an increase in ploidy.

Arabidopsis thaliana has an X-value of 1.2 and a 2C nuclear DNA content of 0.3 pg (Arumuganathan and Earle, 1991). By means of these data, the absolute DNA content of *M. perlarius* plantlets was calculated. DNA content of control *M. perlarius* plants was approximately 5 times higher than for *A. thaliana.* More importantly, the nuclear DNA content of regenerants was about 2 times that of the controls, which is used as evidence for the polyploid status of the regenerated plantlets. Callus material of *M. perlarius* also showed a similar increase in DNA content (data not shown). We therefore presume that the polyploidization *M. perlarius* occurred during callus induction and/or culturing and that the regenerated plants were tetraploid.

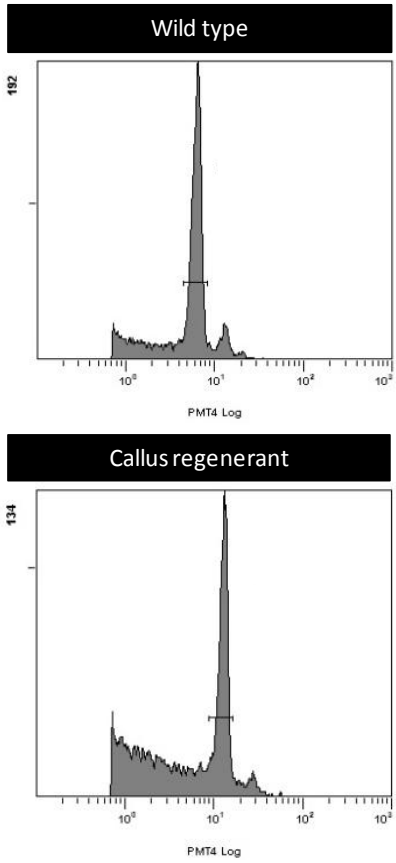

Fig. 2 Histograms of relative fluorescence intensity (log-transformed, PMT4 log) of isolated nuclei from *M. perlarius* wild type shoots and callus-derived shoots.

Table 3 X-values of *Arabidopsis thaliana,* controls and regenerants of *M. perlarius*. Relative DNA content was calculated for *M. perlarius* controls and regenerants based on the 2C nuclear DNA content of *A. thaliana* and X-values. Also the ratio in DNA content between controls and regenerants is represented.

Plant material		X-value	Absolute DNA content (pg)	Ratio control/regenerant
A. thaliana	2C	1.2	0.3	
M. perlarius	Controls	5.9	1.5	2.1
	Regenerants	12.3	3.2	

Morphological characteristics

Table 4 shows the comparison of morphological characteristics between control and callus-derived plants. We found that tetraploid from callus-derived plants showed some morphological alterations. The leaves of tetraploid *M. perlarius* are wider and darker green compared to diploid control plants (Fig. 3a,b). Microscopic imaging and analysis of tetraploid shoots showed a significant difference in stomata length (Fig. 3c,d), which is

consistent with other studies of tetraploid plants (Kaensaksiri et al., 2011; Aina et al., 2012).

Table 4 Morphological characteristics of diploid control and tetraploid callus-derived plants

Ploidy	Leaf length (cm)	Leaf width (cm)	Leaf index (length/width)	Stomata length (µm)
Diploid (Control)	14.50 ± 1.9	6.58 ± 0.7	2.23 ± 0.4	22.10 ± 1.8
Tetraploid (Callus-derived)	15.33 ± 1.0	11.00 ± 0.9	1.4 ± 0.1	33.34 ± 2.3
Significance	ns	*	*	*

* Indicates significant difference ($P < 0.05$) according to two sample t-test and *ns* not significant by the same test

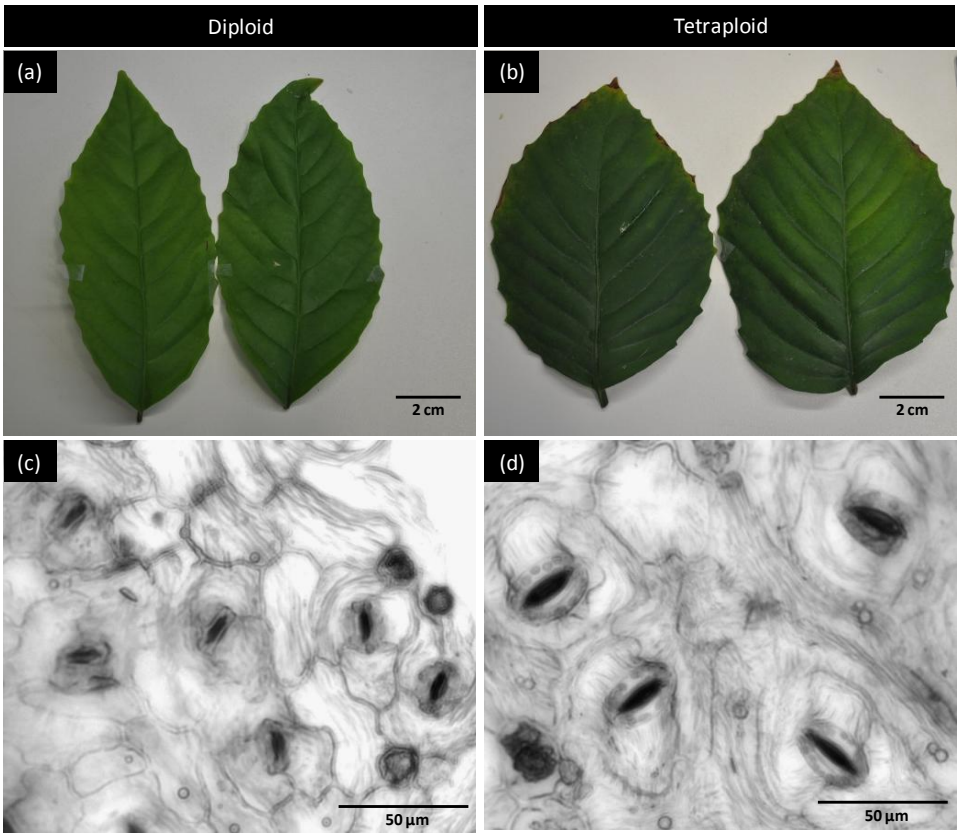

Fig. 3 Leaf characteristics of diploid (a) and tetraploid (b) *M. perlarius* and corresponding stomata (c) and (d) respectively.

Saponin analysis

TLC analysis showed that callus culture from *M. perlarius* did not accumulate saponins (Fig. 4a). Callus extracts contained a compound that produced a large brown spot after TLC

development. Because callus is in direct contact with the sucrose during culturing and sugars react with p-anisaldehyde, we assumed that the brown spot was due to the high sugar content in the medium. Indeed, TLC analysis of different components of the growth medium showed that sucrose produced a similar brown signal as was detected in callus extracts (Fig. 4a).

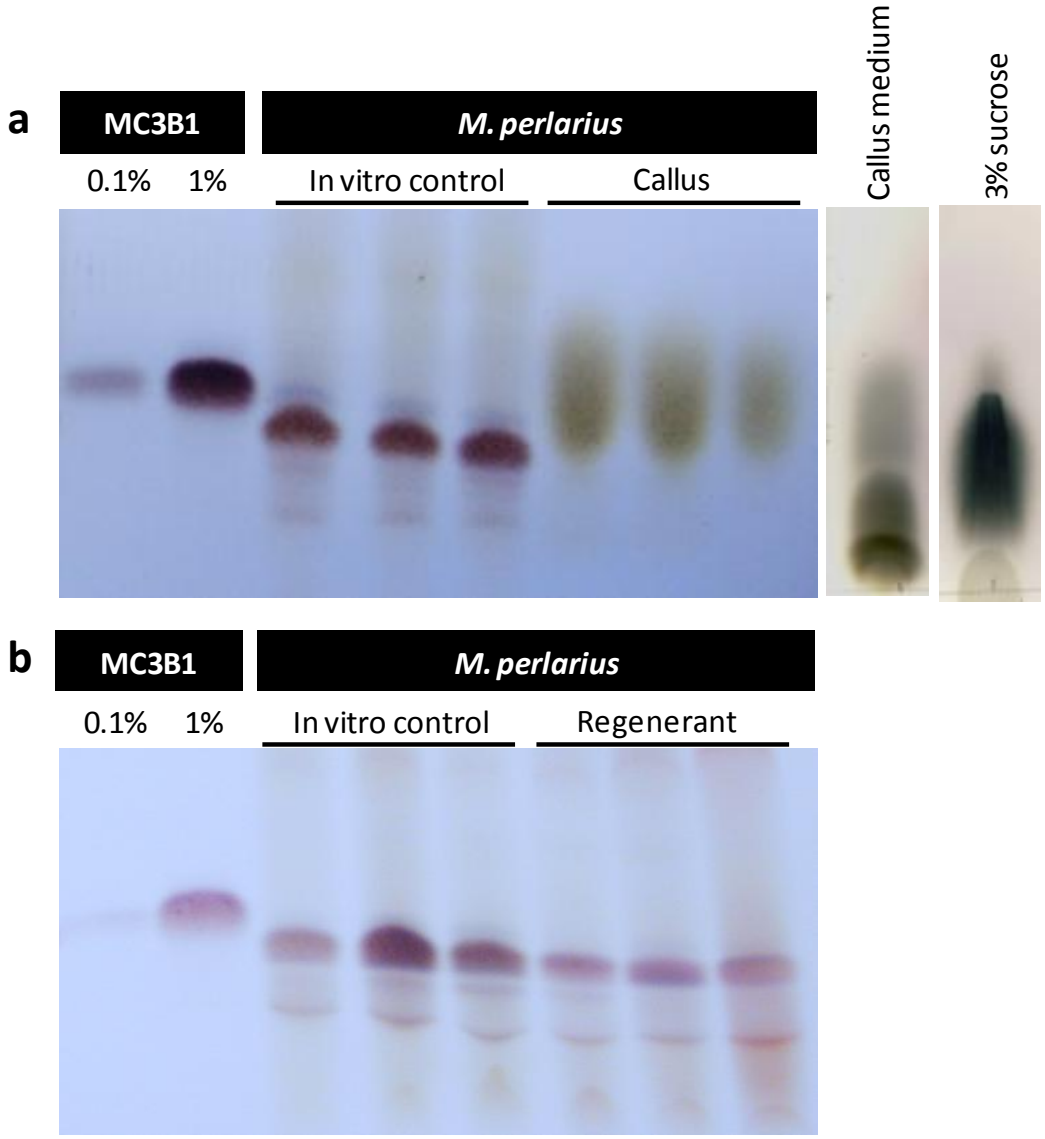

Fig. 4 Saponin TLC profile from in vitro cultures. Callus material (a) from *M. perlarius* did not produce detectable amounts of saponins while the regenerants (b) did contain saponins. Two TLC lanes were included for the culture medium of the callus and 3% of sucrose to explain the background staining with the callus material. MC3B1 is an HPLC purified saponin mixture of *M. lanceolata* saponins and is used here as a reference sample 0,1% and 1% (w/v).

Although calli do not produce detectable amounts of saponin, plantlets regenerated from the same callus material synthesized a substantial amount of saponins, comparable to control plants (Fig. 4b). This indicated that differentiation was necessary for saponin production in *M*. *perlarius*. *M. perlarius* control plants produced one major spot on TLC with an R_f of 0.16. Regenerated plants from callus produced a similar spot with the same retention factor, suggesting that the regeneration process did not influence the saponin production quantitatively. To analyze the saponin composition in extracts from callus regenerated plants we performed LC-MS.

Identification of the saponins was carried out by comparing the molecular weight (MW), retention time (RT), and MS^2 fragmentation pattern with MC3B1 (maesasaponins mixture from *M. lanceolata*) and PX-6518 (maesabalide mixture from *M. balansae*) as reference samples. The results are summarized in Table 5. Typical HPLC profiles of *M. perlarius* from leaf extract of control and callus-derived plants are shown in Fig. 5a-b. Moreover, base peak chromatograms from both control and callus-derived *M. perlarius* displayed similarities with that of the maesabalide standards. Further investigation of the MW, RT and fragmentation patterns confirmed the presence of all maesabalides in *M.* perlarius. Maesabalide I with *m/z* $[M-H]^-$ 1531 showed chromatographic co-elution with the second compound *m/z* 1531 (maesabalide III). In a stretched HPLC run the presence of maesabalide I was confirmed (data not shown). It was, however, not possible to resolve the peaks to determine the relative abundance with great accuracy.

Table 5 LC-MS results

Saponins	*m/z* $(M-H)^-$	RT (min)	*M. perlarius*	
			Control	Callus-regenerant
Maesabalide I*	1531		X	X
Maesabalide II	1509	38.7	X	X
Maesabalide III	1531	38.9	X	X
Maesabalide IV	1509	40.0	X	X
Maesabalide V	1573	41.3	X	X
Maesabalide VI	1551	42.0	X	X

X = identical molecular weight, retention time and MS^2 fragmentation pattern; * Maesabalide I showed chromatoghrapic co-elution with maesabalide III and an identical *m/z* and could not be separately identified.

According to Dhawan and Lavania (1996), polyploidization may alter the secondary metabolite in qualitative and quantitative manner. Because callus derived *M. perlarius* shoots were tetraploid, therefore we anticipated conspicuous changes on chemical profile of metabolic products. However, LC-MS analysis showed that seed-derived and regenerated *M. perlarius* produced identical saponin profiles.

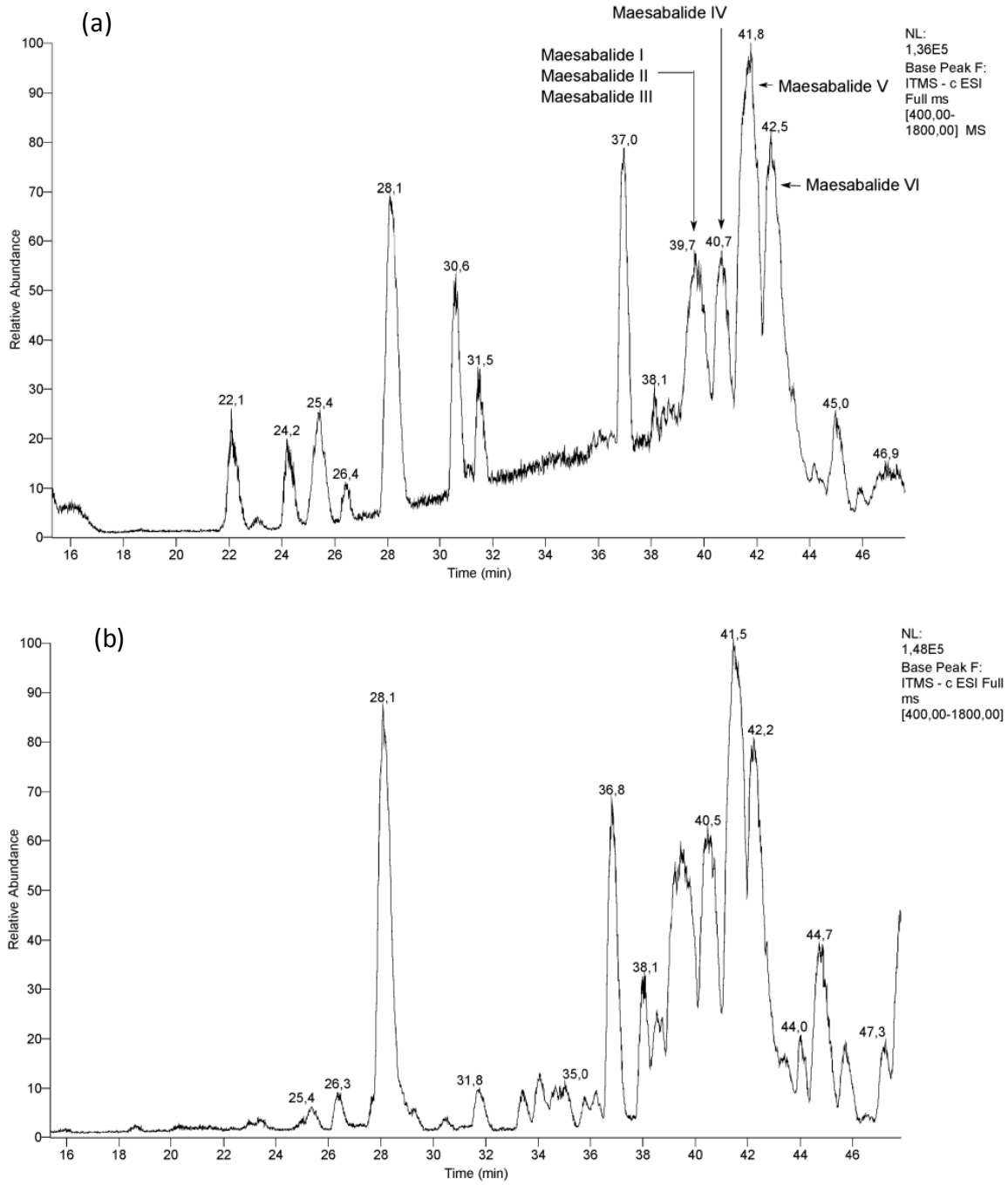

Fig. 5 Base peak chromatogram of the leaf extract of *M. perlarius* control plant (a) and callus-derived plant (b).

A careful comparison of the HPLC profiles of diploid control and tetraploid *M. perlarius* did not reveal qualitative differences of known maesabalides, suggesting that the biosynthesis pathway of saponins is robust and withstands genome doubling. However, we did observe several highly mobile products with low retention times in the extracts from diploids, which were absent in the tetraploid plants. These observations indicate that in contrast to saponins, the production of other, unidentified secondary metabolites, are reduced upon polyploidization. To determine the relative abundance of the saponins in diploid and tetraploid plants, we performed the integration of the base peak chromatogram of each individual peak corresponding to known maesabalides. The relative abundance of all maesabalides from tetraploid *M. perlarius* showed no significant differences with those of control plants (Fig. 6).

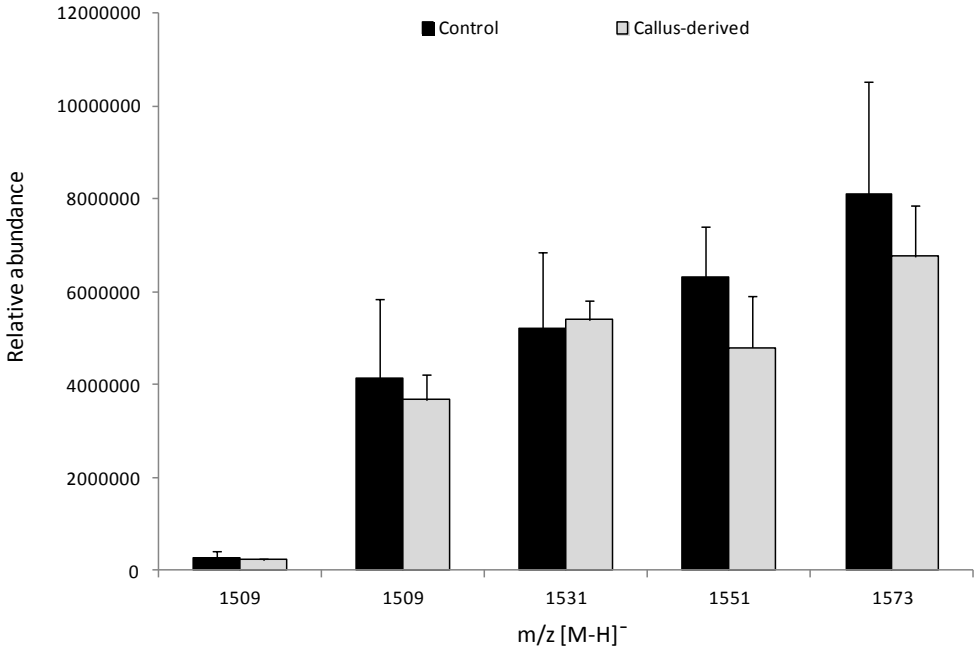

Fig. 6 Relative abundance of individual maesabalides present in *M. perlarius*. Vertical bars indicate the standard deviation of the mean of three replicate experiments.

3. Discussion

The regeneration of callus has successfully been used for mass propagation and for obtaining useful variants in several medicinal plant species (Rout et al., 2000). Calli of *M. perlarius*, cultured during 1 year, maintained competence to form shoots. It has also been

reported that the combination of two cytokinins such as BA and kin could be used to increase indirect shoot regeneration of medicinal plants (Mohanty et al., 2008; Shriram et al., 2008; Baskaran et al., 2011). Therefore, 15 different combinations of BA and kin were tested for callus regeneration. However, none of these hormone combinations was effective in inducing shoots from *M. perlarius* calli. In contrast, a combination of TDZ and NAA was effective in shoot regeneration for *M. perlarius*. Callus regeneration protocols have been described for many other medicinal plants and effective hormone combinations were clearly dependent on the plant species used. For example, a combination of BA and kin was most effective for callus regeneration of *Helicteres isora* (Shriram et al., 2008), kin and NAA for *Lonicera macranthoides* (Wang et al., 2009) and BA and NAA for *Allium chinense* (Yan et al., 2009).

Callus material of *M. perlarius* did not contain sufficient amounts of saponin for detection on TLC. Similar to our findings, cell suspension culture of *Glycyrrhiza glabra* also did not produce glycyrrhizin, a sweet oleanane-type triterpene saponin (Hayashi et al., 2004). It has been generally accepted that production of secondary metabolites is usually low in undifferentiated fast growing tissue (Banthorpe et al., 1986; Bourgaud et al., 2001; Collin, 2001). Although there are some reports in which cell cultures are successfully used for the production of secondary metabolites (Bonfill et al., 2011; Malik et al., 2011; Grover et al., 2012; Wang et al., 2012), there are also reports that describe a decrease in secondary metabolite production in de-differentiated tissue (Baiza et al., 1998; Pinol et al., 1999).

Regenerated shoots from callus had maintained the capacity to produce saponin despite the absence of saponin biosynthesis in callus culture. The relative abundance of the TLC detected saponin spot was similar to that detected in seedling-derived plants.

In contrast to in vitro propagation through axillary and adventitious shoot regeneration that maintained the same ploidy level as reported in Faizal et al. (2011) and in chapter 2, callus regeneration in *M. perlarius* resulted in tetraploid plants. It is generally believed that tetraploid cells have a slightly shorter cell cycle, which in the long run leads to elimination of the slower diploid cells. The accelerated growth behavior does not exceed beyond the tetraploid level as we did not find callus with higher ploidy levels. In addition, an altered chemical profile of secondary metabolites has been reported (Lavania, 2005; Lavania et al., 2012). The multiplicity of allelic interactions, which is possible after genome duplication, can influence the relative abundances of different key enzymes and this may

alter preference of the biosynthetic routes followed. This phenomenon may result in an altered accumulation of secondary metabolites.

It has also been reported that polyploidization may lead to an increase in secondary metabolite production through chromatin remodeling and epigenetic deregulation, which are induced by in vitro cultivation and or polyploidization (Riddle et al., 2010; Miguel and Marum, 2011). For example, the increased of terpenoid indole alkaloid content in *C. roseus* lines was correlated to higher expression level of genes involved in its biosynthetic pathway of tetraploid lines (Xing et al., 2011). In *M. perlarius* however, an increased of gene copy number did not influence maesabalide production. Hence, the regulatory circuitry controlling saponin production is robust and probably relies on feed back control to maintain appropriate biosynthesis gene expression levels.

In conclusion, in vitro callus induction and regeneration as we reported here is prone to ploidy change. In this study, we also present evidence that genome duplication had no qualitative or quantitative effect on saponin production.

4. Materials and methods

Plant Material

Plant material that was used in this work was derived from a germinated seed that was clonally propagated following the in vitro propagation protocol previously described (Faizal et al., 2011).

Callus induction

Leaves of in vitro grown *M. perlarius* were used as explants for callus induction. The leaves were isolated and cut into slices of approximately 0.5 x 0.5 cm^2 and were then put on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham) and 3% (w/v) sucrose (pH 5.8). For callus induction, auxins and cytokinins were added to the basal medium in different combinations and the effect of different hormones and combinations of hormones on callus induction was studied. The following growth regulators were used in the given concentration; 2.7 µM NAA, 4.4 µM BA, 5 µM 2,4-D and 4.6 µM kin. Leaf material was incubated at 25 °C in continuous darkness on Petri dishes (3 leaf slices/dish). The

experiment was performed in triplicate with nine leaf slices per repeat. Callus formation was scored 6 weeks later. A single callus per leaf explant was put on fresh medium with the same composition as for the callus induction. Calli were subcultured every month.

Callus regeneration

The calli of *M. perlarius* were grown and maintained on medium with 5 µM 2,4-D. For regeneration, one year old calli were transferred to Petri dishes with MS basal medium supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose and different concentrations of hormones, either alone or in combinations (9 calli/dish). The following phytohormones were used in the given concentrations; 0.4 µM, 2.2 µM and 4.4 µM BA and 0.5 µM, 2.3 µM and 4.6 µM kin. Callus on regeneration medium was incubated at 25 °C with a 16/8 h light/dark photoperiod (32.7 ± 3.23 µmol m^{-2} s^{-1}) provided by warm white fluorescent light (Osram, Germany). Six weeks after incubation on medium with BA and kin, the calli were split in two; half of the callus was placed on fresh medium with the same concentration of BA and kin and the other half was placed on medium with 4.5 µM TDZ and 0.05 µM NAA. The experiment was performed in triplicate with nine calli per replicate. Shoot induction was scored 17 weeks after the start of the experiment. Regenerated shoots were isolated and transferred to basal MS medium lacking growth regulators and supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar for elongation and rooting in single step. Plantlets formed roots after 3 weeks in culture were then acclimatized according to Faizal et al. (2011).

Ploidy analysis: flow cytometry

Flow cytometry was performed according to protocol described in chapter 2.

Morphological analysis

In order to compare the control and callus-derived plant, especially diploid and tetraploid plants, we evaluated length and width of fully mature leaves and leaf index (length/width) from 3 independent replicates. Each evaluation comprised 10 leaves. For stomatal analyses, 3 fully mature leaves from each plant were sampled. The abaxial epidermises of leaf were peeled and were mounted on slides. Images were captured by using an inverted Olympus IX81 microscope equipped with an XM-10 (Olympus) camera. The stomata length was

measured from the captured images obtained with a 20x objective lens using CellM™ software (Olympus).

Saponin analysis

Thin layer chromatography

Saponin extraction and TLC analyses has been described in Chapter 3.

LC-MS analysis

Oven-dried leaf material from 3 independent greenhouse plants regenerated from callus and 3 independent control plants were grounded and extracted with MeOH 50% (v/v) by sonication, after which the extract were dried under reduced pressure. HPLC analysis of methanol 80% solutions of the *M. perlarius* (10 mg/mL) and the semi-pure extract of *M. balansae* (5 mg/mL) was performed according to the method of (Theunis et al., 2007) and was also described in chapter 3.

Statistical analysis

Data were analyzed using one-way ANOVA and the comparison of the mean callus regeneration was contrasted using Duncan's multiple range test. Two sample t-test was used to compare the morphological characteristics of control and callus-derived plants. The Mann-Whitney U test allowed us to compare the difference of relative saponin abundance between control and callus-derived plants. All statistical analyses were performed at the level of *P*-value less than 0.05 using SPSS 18.0 (SPSS Inc. USA).

Acknowledgements

We thank the BOF (special research fund No. 01WI0111, Ghent University) for A.F. PhD. fellowship. This research is supported by a research grant G.0014.08 offered by the Flemish Funding Agency for Scientific Research (FWO).

8 Conclusions and perspectives

Cover art: “I will beat him”

8

Conclusions and perspectives

Saponins are part of a diversity of secondary metabolites that have been clearly demonstrated to play a role in the adaptation of plants to their environment. The production of these secondary molecules may be part of the response to external factors including various biotic and abiotic stimuli. In addition, their function as anti-microbial has driven the interest to fully exploit their potential in medicinal applications.

The importance of *Maesa* species as medicinal plants

Maesa species are a group of saponin producing plants, which have interesting medicinal properties and promising pharmaceutical applications. Medicinal plants have been used for thousands of years as the main sources of therapeutic agents and even today a large proportion of the world's population still rely on plants as a primary source for traditional medicine. Here, four *Maesa* species, *Maesa argentea*, *M. balansae*, *M. lanceolata*, and *M. perlarius,* have been selected based on previous ethnopharmacology investigations. Further evaluations using bioassay-guided fractionation procedures lead to identification of saponins with a myriad of biological activities such as virucidal, fungicidal, insecticidal, and molluscicidal activities (Sindambiwe et al., 1998; Apers et al., 2001). One of the mentioned species, *M. lanceolata*, possesses antiangiogenic activity and could therefore be used as a potential cancer chemotherapeutic agent (Apers et al., 2002; Foubert et al., 2012). As we know, cancer remains for a major cause of death among the populations in the world. However, the potential for discovery of a new anticancer agent from plants appears to be relatively low compared to other types of organisms (Newman and Cragg, 2012). This is because plants have not been as productive as other type organisms in yielding structurally diverse compounds (Kinghorn et al., 2011). Despite this paucity, the anticancer agents from plants are still of interest today since they have frequently shown novel mechanisms of action.

M. balansae saponins have been investigated because of their strong anti-leishmanial activity (Maes et al., 2004). *M. argentea* and *M. perlarius* have not yet been investigated in detail, however, they also produce a saponin mixture with anti-leishmanial actions (Foubert et al., 2008; Vermeersch et al., 2009). Knowledge of the regulation of saponin biosynthesis from these plants will be critical for optimization of saponin production and for future medicinal purposes.

In vitro conservation of medicinal plants

Many additional important medicinal plants are in supply crisis or near extinction. For example wild ginseng, one of the most valuable medicinal plants, now is rarely available and the ginseng roots in the market are mostly collected from farms cultivating ginseng in fields (Wu and Zhong, 1999). To circumvent the problems with conventional propagation of *Maesa* spp., we therefore established an effective means for in vitro propagation of medicinal plants that produce pharmaceutically interesting saponins. The use of in vitro propagation not only will reduce pressure on natural population, but also will become one of the most effective ways of conservation (Rout et al., 2000). In addition, plant tissue culture can provide plant materials with more controllable production in terms of quality and quantity, while the culture conditions and process variables are more easily optimized (Wu and Zhong, 1999). This is important for production of uniform compounds required for the preparation of standard formulations.

In addition to in vitro materials, we also evaluated the content of saponin from *Maesa* grown in greenhouse conditions. We observed that the saponin content varied depending on organ and physiological age of the plants. In addition, the production of saponin was very stable and not affected by treatment with hormones and/or elicitors. As a conclusion, *Maesa* saponins are constitutively produced in plants and the level of these compounds in plants is mainly affected by the developmental or physiological stage. We discovered that the correct time of harvesting, the total biomass and the age of harvested organs were critical to extract the maximum level of saponin.

Strategies developed for saponin modulation in *Maesa*

Next to the classic plant tissue culture, this work contributed to the COMBIPLAN project, a collaborative effort of three research groups, the department of Plant Systems Biology (Ghent University/VIB) the department of Pharmaceutical Sciences (University of Antwerp) and the department of Plant Production (Ghent University) to establish a combinatorial biosynthesis platform in plants with the triterpene saponins as the target. The aim was to facilitate semi-rational combinatorial engineering of the biosynthesis of existing and novel secondary metabolites in plant cell tissue culture. Using Fourier transform ion cyclotron resonance mass spectrometry, previously undetected saponins have been identified, but so far no truly novel saponin compounds have been produced in *M. lanceolata*. This shows that

the engineering of saponins is not straightforward (Pollier, 2011). Taken together, this may also indicate a complicated regulation process of saponin biosynthesis, which is dependent on other metabolic pathways as sources of precursors and intermediates. To avoid these secondary interactions, a heterologous host system, for example using a microbial production platform, should be envisaged as production vehicle.

Efforts to modulate saponin production through combinatorial biosynthesis in *M. lanceolata* hairy roots did not prove successful. Although hairy root induction is perfectly suitable for the screening of different constructs and analysis of small samples of transgenic material, it may not be suitable for the expression of leaf-derived cDNA sequences. This prompted us to switch our focus on the development of an *Agrobacterium tumefaciens*-mediated transformation protocol. Here, we show that agroinfiltration can be used not only for a transient expression system, but also as an alternative approach to regenerate stably transformed plants. The optimized protocol may allow the screening of candidate genes which are suspected to enhance saponin production or drive production towards one specific class of saponins. Importantly, this technique can also be applied to other plant species that are permissive for agroinfiltration.

One of the remaining challenges for complex compounds in many plants is that they are often only available in very low concentrations. For this, elicitor treatment was reported to have successfully increased yields of bioactive saponins in various plant species and experimental systems (Yendo et al., 2010). A whole array of potential elicitors has been tested on hairy roots (Lambert, 2011) and in vitro shoot culture and none of the substances were capable of effectively inducing saponin synthesis in *Maesa* spp.

An alternative method involves the overexpression or the silencing of regulatory genes which may lead to a higher accumulation of the desired compounds. Since the product of β-amyrin synthase is one of the key precursors of the triterpene saponin pathway, overexpression of β-amyrin synthase potentially leads to increased saponin production. Indeed, we describe how overexpression of *M. lanceolata* β-amyrin synthase (*Ml-b*AS) enhances saponin accumulation. Interestingly, in some transgenic lines it also caused co-suppression resulting in a reduction in the production of saponin. This indicates that the expression of *Ml-b*AS in *M. lanceolata* directly correlates with saponin production. It also provides a better understanding on how saponins are regulated in plants. Additionally, these overproducing lines may turn to be a suitable host for functional studies on other

saponin biosynthesis candidate genes isolated during COMBIPLAN project. Further analysis should be performed whether the overexpression of *Ml-b*AS may lead to different accumulation of maesasaponin subclasses. This will be important for future application, for example selective material for drug testing since the subclasses of maesasaponin have demonstrated different bioactivities.

Finally, we reported the spontaneous polyploidization in *M. perlarius*. This could be an interesting tool to produce superior plant in terms of saponin production. In addition, polyploidization may have qualitative effects as many developmental processes are deregulated in polyploid plants. However, we showed that saponin production is not qualitatively changed by a genome-doubling event. This indicates that in vitro cultivation developed in this PhD work could be used for standardization of plant material since the content of saponin was not qualitatively and quantitatively affected.

In conclusion, the possible application of in vitro-controlled conditions developed for unfamiliar *Maesa* species will contribute to the domestication of wild plant species and intensification of secondary metabolite production. Additionally, this research provides information to determine the most appropriate harvesting stage for rational exploitation of natural plant resource and to avoid poor quality material. This will facilitate the quality control of the material and plant-derived product. Taken together, several strategies developed in this PhD project resulted in establishment of a platform for optimization of saponin production from medicinal *Maesa* species. Furthermore, the identification and development of saponins guide us to understand the regulation of secondary metabolites in plants. The abundance and stable production of saponins in *Maesa* are not only important for medicinal application, but also fits with a role of these compounds for plant protection. The fact that saponins have been reported to show rapid and high activities against aphids and other pest insects (De Geyter et al., 2012a; De Geyter et al., 2012b) makes them potentially valuable for plant-based pesticides, which may be more environmental-friendly as compared to synthetic products. However, more research is necessary to develop this information for crop protection purposes since we did not perform any pathogenic tests.

Summary | Samenvatting

Cover art: composition of about 50 TLC images generated during this work

Summary

Saponins are secondary metabolites that play an important role as a defense mechanism against herbivores and pathogens (**Chapter 1**). The wide-spread occurrence in plants as well the potential for pharmaceutical application has lead to saponin extraction and identification in numerous species.

The overall goal of this thesis was to develop strategies to build a platform for secondary metabolite studies in tropical medicinal plant species. Four species (*Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius*) were selected based on previous ethnobotanical studies. Further investigation reported that these species produce a mixture of pharmaceutically interesting saponins with virucidal, molluscicidal, cytotoxic, haemolytic, anti-leishmanial and antiangiogenic activity. To facilitate these studies, we have established an in vitro propagation method for four selected *Maesa* species (**Chapter 2**).

Using in vitro grown material together with greenhouse-grown plants, we investigated the saponin production in more detail. The importance of factors possibly influencing saponin content, including organ type, age and phytohormone treatment were also studied (**Chapter 3**). We identified that *Maesa* saponins are constitutively produced in plants and the level of these compounds in plants is mainly affected by the plants' developmental and physiological stage.

To modulate saponin production, a combinatorial biosynthesis approach is also proposed in *M. lanceolata* (**Chapter 4**). Using a cDNA-AFLP tool, we have selected candidate genes involved in saponin biosynthesis from other saponin producing plants. These genes were subsequently used for transformation of *M. lanceolata* leaf discs with *Agrobacterium rhizogenes*. Unfortunately, this approach did not prove to be successful.

Despite that hairy root was a good system for the screening of different constructs and analysis of transgenic material, our findings showed that saponin content was much lower in hairy roots compared to shoots. This could be a limiting factor to modulate saponin production using hairy root materials. Therefore, we decided to use shoot materials and set up an *A. tumefaciens*-mediated transformation protocol for *M. lanceolata* (**Chapter 5**). Both a transient expression and stable transformation method were developed using

agroinfiltration to allow the screening of candidate genes which are suspected to enhance saponin production or drive production towards one specific class of saponins.

Modulation of saponin production can also be achieved by overexpression of key enzymes that interfere with saponin biosynthesis pathway. To investigate this, we have isolated *M. lanceolata* β-amyrin synthase (*Ml-b*AS) and subsequently overexpressed this gene in *M. lanceolata*. We demonstrated that this approach enhanced saponin accumulation, although in some individuals it causes a co-suppression resulting in a lower production of saponin (**Chapter 6**).

Finally, we noticed that shoots regenerated from *M. perlarius* callus were tetraploid (**Chapter 7**). We took advantage from the fortuitous finding to study the impact of polyploidy on saponin production. Our results showed that saponin production was not altered by the increased ploidy, in contrast to other, non-identified, metabolites. These results suggest that saponin biosynthesis in *M. perlarius* is a robust process, unaffected by doubling of gene dose.

Samenvatting

Saponinen zijn secundaire plant metabolieten die een belangrijke rol spelen bij de afweermechanismen tegen herbivoren en pathogenen (**Hoofdstuk 1**). Hun wijdverbreid voorkomen in het plantenrijk en hun potentiële gebruik in geneesmiddelen, leidde tot saponine-extractie en –identificatie in verschillende species.

Het algemene doel van deze thesis was het ontwikkelen van strategieën waarbij een platform voor de studie van secundaire metabolieten in tropische, geneeskrachtige planten wordt gecreëerd. Vier species (*Maesa argentea, M. balansae, M. lanceolata* and *M. perlarius*) werden in eerdere ethnobotanische studies geselecteerd. Vervolgonderzoek wees uit dat deze species een mengsel van farmaceutisch interessante saponinen produceren. Deze saponinen omvatten metabolieten die gebruikt kunnen worden als bestrijdingsmiddelen tegen weekdieren of met antivirale, cytotoxische, haemolytische, antileishmania en anti-angiogene eigenschappen. Om verdere studies te vergemakkelijken hebben we een *in vitro* vermenigvuldigingsmethode voor de vier geselecteerde *Maesa* species op punt gesteld (**Hoofdstuk 2**).

Via *in vitro* material en de vergelijking met serreplanten, bestudeerden we de saponine-productie in meer detail. Het belang van factoren die de saponine-inhoud beïnvloeden, daaronder verstaan we het orgaantype, de ouderdom en plantenhormoon-behandelingen, werd ook bestudeerd (**Hoofdstuk 3**). We vonden dat *Maesa* saponinen constitutief worden geproduceerd in planten en dat de hoeveelheid van deze verbindingen voornamelijk beïnvloed wordt door de ontwikkelingstoestand en –leeftijd van de planten.

Daarenboven stellen we een combinatorische benadering van de biosynthese in *M. lanceolata* voor waarbij we hopen de saponine-productie te wijzigen (**Hoofdstuk 4**). In een cDNA-AFLP selecteerden we kandidaat-genen betrokken bij saponine-biosynthese in andere saponine-producerende species. Deze genen warden vervolgens gebruikt voor de transformatie van *M. lanceolata* bladschijven met behulp van *Agrobacterium rhizogenes.* Helaas leidde deze aanpak niet tot succes.

Ondanks het feit dat de hairy roots een goed screeningsysteem voor verschillende constructen en voor de analyse van transgeen materiaal bleken, toonden onze bevindingen

aan dat de saponine-inhoud veel lager was in deze weefsels in vergelijking met scheuten. Dit kan een limiterende factor zijn voor de modulatie van saponine-productie in hairy roots. Om die reden werd besloten scheutmateriaal te gebruiken en een *A. tumefaciens*-gemedieerd transformatieprotocol werd opgesteld voor *M. lanceolata* (**Hoofdstuk 5**). Via agroinfiltratie warden zowel transiënte expressie als stabiele transformatie bekomen zodat screening van kandidaatgenen die verwacht worden de saponine-productie te verbeteren of te sturen in de richting van specifieke saponineklassen.

Veranderingen van de saponine-productie kunnen ook bekomen worden via het tot overexpressie brengen van sleutelenzymen die interfereren met de biosynthese. Om deze strategie te onderzoeken, isoleerden we *M. lanceolata* β-amyrine synthase (*Ml-b*AS) en brachten we dit gen tot overexpressie in *M. lanceolata*. We toonden aan dat in deze werkwijze de saponine-accumulatie verhoogt, hoewel in sommige individuen cosuppressie resulteerde in een verlaagde saponine-productie (**Hoofdstuk 6**).

Tot slot werd geconstateerd dat scheuten die genereren van *M. perlarius* callus tetraploid waren (**Hoofdstuk 7**). Gebruikmakende van deze bij toeval bevinding, bestudeerden we de impact van polyploïdie op de saponine-productie. Onze resultaten toonden aan dat de saponine-productie niet gewijzigd werd bij stijgende ploïdie, dit in tegenstelling tot andere niet-geïdentificeerde metabolieten. Deze resultaten tonen aan dat de saponine-biosynthese in *M. perlarius* een robuust proces is, dat niet beïnvloed wordt door genverdubbelingen.

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Thanks

I would like to thank the almighty Allah for the uncountable blessing that He has bestowed upon me. I dedicate this thesis to my parents (Mide and Habi). I wish I could see their happy smiling face. Hope Allah will grant them the best place in heaven.

I always thought that writing the acknowledgements would be the easiest and most relaxing part of the whole PhD thesis. I was wrong. In fact, it took me about the same time as writing other chapters in this thesis. Although the main objective of this section is to express my gratitude, I realized that this is also my only chance to tell a bit more about my experiences during my study. I still remember the day I stepped into the lab. It was the New Year reception, which meant that I was welcomed by all the faculty members. That was the warmth feel in a snowy winter. When I started my PhD, I thought 4 years would be an extremely long time to stay away from home, family and friends, confined in Ghent, a small city of Belgium that I had never heard about. Again, I was wrong. During the last 4 years, I challenged my personal limits in many ways, I achieved important aims, expanded my geographic, social and scientific knowledge and of course, had fun doing so, but all these would not have been realized without the contribution of the wonderful people who made this experience the most extraordinary to me.

My sincerest appreciations go to my promotor Prof. Danny Geelen. Thank you so much for welcoming me in your lab. Thank you for your support when I felt frustrated, especially during the first year. Your knowledge and advice helped me to stay on track. Your guidance lets me improve my theoretical and practical skills. You were almost available every time I need your help and I could always expect your email during the weekend or even at midnight.

My gratitude goes out as well to the reading and examination committee: Prof. Peter Bossier, Prof. Alain Goossens, Prof. Godelieve Gheysen, Prof. Luc Pieters, Prof. Marie-Christine Van Labeke and Prof. Monica Hofte. Thank you for sharing your expertise and knowledge. Your critical comments and suggestions toward my thesis have honed my research and made my writing a little less amateur.

I am greatly indebted to School of Life Sciences and Technology, Institut Teknologi Bandung and the Directorate of Higher Education, Ministry of Education and Culture, Republic of Indonesia for giving me the opportunity to pursue my dreams in becoming a researcher. A special thanks to Prof. Intan Ahmad and Dr. Maelita Moeis who opened the way by initiating the "Presidential Scholar Fund" (PSF) program; to all PSF members, Intan Taufik, Sidrotun Naim, Elvi Restiawati, Dian Rosleine, and Azzania Fibriani; I am also thankful to the present dean, Prof. Tati Subahar; Plant Science and Biotechnology Research Group, Dr. Iriawati and Dr. Rizkita for all the supports; Dr. Gede Suantika, thanks for the information and your encouragement to study in Ghent University. I wouldn't have known Ghent without you being the pioneer graduated from this University.

I am also thankful to Ghent University for giving me the opportunity to experience the best learning processes, interesting courses, enjoyable conferences, and of course with support from its "Special Research Fund" (BOF), I could finalize my PhD.

Next, I will list everybody I met while working in the In vitro biology and horticulture lab. I hope I won't forget anybody because the list is very long. Many are close friends, whose enthusiasm, interest and support in this project have given me the motivation to realize this achievement. Ellen Lambert, my partner in crime. Thank you for your help in the lab. Thank you for sharing experiments, discussion and for the great teamwork. Your contribution to my work was technically the most important as your name is almost in all my experimental chapters; Nico De Storme, although you are not really a football lover, at least we can always discuss many things about football. Big thanks also for your help with flow cytometry. Good luck for your post-doc application and I hope you will become one of the leading authority in the field of plant sexual reproduction ☺; Inge Verstraeten, thank you for being such a nice friend and also for being my Dutch translator. I hope you will enjoy your next journey in USA; Mandy Rasmussen, aka Mega Mandy, aka Razda. You were a big help in the writing of my thesis. Thank you for correcting typos, grammar and for improving my language style. Being friend to a native English speaker is a great advantage to help me improve my writing skills, and you are also an awesome friend; Linda Zamariola, "il mio migliore amico italiano". Grazie per tutto…. Good luck with your *Brassica*. Next year will be yours…; Machteld, thanks for hosting me in your place the first time I arrived in Ghent. You are always very helpful; Norhayati Daud, it was a pleasure to work with you. Without your big contributions, we couldn't get rid out that *Jatropha* story; Christophe Petit, I used to call

you a Professor because of your expertise in many things. You are an expert in multimedia, network, greenhouse and all technical details; Patricia and Ellen Van Gysegem, thanks for the technical supports in the lab; Katrijn, Mary, Koen, Emmy, Thea, Mieke, Annelies, Thuc, Farzaneh, Yannick, Valerie, Katrien, Sara, Sami, Steffi, Killian, Mouna, Ester, Joan, Stephanie, Ahmed, Luca, Andrea, Hannes, Brecht and (who else...?).

All people involved in the COMBIPLAN-project: Prof. Alain Goossens, Jacob Pollier, Lander, and Tessa Moses, from VIB Department of Plant System Biology – Ghent University and Prof. Sandra Apers, Prof. Luc Pieters, Kenn Foubert, Mart and Taposh from the Department of Pharmaceutical Sciences (University of Antwerp). Special thanks to Jacob and Kenne, without your help and input, this thesis wouldn't be what it is.

Ann Djudzman and Francis Van De Walle, thanks for your hospitality and your kind help with everything.

To all my Indonesian friends in Ghent, it was a pleasure to have such a big family with you. I will never forget the time we spent together. Special thanks to Hanifah Siregar who has designed a nice cover for my thesis.

Pak Baktiar, Kak Lely, Kak Miya, Ko Juliando, Ci Lanny, Mba Maya and Mba Desi; Thanks for your supports and because of you, I didn't forget the taste of Indonesian food.

"Gapleh" team (Pande, Romi, Nanang, Wahyu), thanks for the relaxing-moment ☺, it was fun.

Numerous thanks to Dr. Agus Dana Permana. Thanks for your unlimited supports to me. Also to all members of TOBI (Tim Olimpiade Biologi Indonesia). I am going to join you again guys.

I would like to present my sincere thankfulness to my brothers and sisters, Kak Hapipa, Kak Bahera, Kak Marwa, Kak Jamal, Kak Rahman, Kak Hamid, Kak Suhaemi, and Kak Sudarmi. Thanks for your supports from afar. Additonally, thanks to my lovely papa Saifuddin, mama Saida, and Bro Yoga, your support and pray has helped me a lot.

The last and the most important, my K3 (Kiky, Kynan and Kika). Having you is the best thing which has ever occurred to me in my entire life! My wife Kiky, I could not be able to finish this work without your support. Thank you for being my best partner and for your appreciated sacrifices. Thank you my dynamic duo, Kynan and Kika. Thank you for being good kids while I was study. You are the reason why I always keep the spirit to finish things that I have started.

Finally my PhD is over. Yeaaahhhhhh!

Bedankt!

Faizal, Ghent 2013

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PUBLICATIONS

Ahmad Faizal and Danny Geelen. **Saponins and their role in biological processes in plants**. *Phytochemistry Reviews*. Under review

Norhayati Daud, Ahmad Faizal, and Danny Geelen. 2013. **Adventitious rooting of *Jatropha curcas* L. is stimulated by phloroglucinol and by red LED light.** *In Vitro Cellular & Developmental Biology-Plant.* Doi**:** 10.1007/s11627-012-9486-4

Ahmad Faizal, Kenn Foubert, Ellen Lambert, Nico De Storme, Magda Claeys, Sandra Apers and Danny Geelen. 2013. **Saponin production is not qualitatively changed upon callus regeneration in the medicinal shrub *Maesa perlarius***. *Plant Growth Regulation*. Doi: 10.1007/s10725-012-9776-1

Kenn Foubert, Annelies Breynaert, Mart Theunis, Rita Van Den Bossche, Guido R.Y. De Meyer, André Van Daele, Ahmad Faizal, Alain Goossens, Danny Geelen, Edward M. Conway, Arnold Vlietinck, Luc Pieters, and Sandra Apers. 2012. **Evaluation of the anti-angiogenic activity of saponins from *Maesa lanceolata* by different assays**. *Natural Product Communications*, 7:1149-1154

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OTHER SCIENTIFIC COMMUNICATIONS

Ahmad Faizal and Danny Geelen. (2012). **Genome doubling does not affect the saponin production from callus-derived shoots of *Maesa perlarius*.** Oral presentation at 5th International symposium breeding research on medicinal and aromatic plants. Vienna (Austria) 18-20 June 2012.

Ahmad Faizal and Danny Geelen. (2011). **Agroinfiltration of the medicinal plant *Maesa lanceolata* leaves for transient gene expression assay and stable transformation**. Poster talk at 7th In vitro culture and horticulture breeding symposium. Ghent (Belgium) 18-22 September 2011.

Ahmad Faizal and Danny Geelen. (2011). **Agroinfiltration of the medicinal plant *Maesa lanceolata* leaves for transient and stable transformation**. Poster presentation at Plant Transformation Technologies II. Vienna (Austria) 19-22 February 2011.

Ahmad Faizal and Danny Geelen. (2010). **Genetic engineering of the medicinal plant *Maesa lanceolata***. Poster presentation at New Biotrends in green chemistry. Dortmund (Germany) 1-2 December 2010.

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Additional publications

Adventitious rooting of *Jatropha curcas* L. is stimulated by phloroglucinol and by red LED light

Norhayati Daud • **Ahmad Faizal** • Danny Geelen

An efficient root induction system has been established for in vitro-regenerated *Jatropha curcas* L. shoots. Callus formation on shoots transferred to auxin containing medium was found to be a prominent and recurrent problem for rooting of in vitro-cultivated *J. curcas*. In particular, the type of auxins and cytokinins applied in the culture media were shown to strongly influence the severity of callus formation. Shoots cultivated on meta-methoxytopolin riboside (MemTR) were free of callus and produced elongated stems and well-developed leaves in comparison to the cytokinins benzyladenine, zeatin, and thidiazuron. Subsequent root induction experiments were performed with shoots precultured on MemTR-containing medium. Shoots were excised and transferred to Murashige and Skoog (MS) medium supplemented with different concentrations of indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), and α-naphtaleneacetic acid (NAA). The induction of excessive callus formation was avoided only on IBA-containing medium. The optimum rooting medium with good root induction (35%) and 1.2 roots per shoot contained half-strength MS salts supplemented with 2.5 µM IBA. The same medium supplemented with 0.25% (w/v) activated charcoal produced 46% rooted shoots. Further improvement of rooting was obtained by transferring in vitro grown shoots to woody plant medium containing phloroglucinol (PG). In the presence of 2.5 µM IBA and 238 µM PG, 83% of the shoots rooted with on average 3.1 roots per shoot. We also analyzed the impact of light quality on the rooting capacity of Jatropha in vitro grown shoots. In general, light-emitting diodes (LEDs) light sources were less efficient for root induction. Red LED light provided the most favorable growth conditions, inducing a rooting response in 65% of the shoots, which produced on average 5.5 roots per shoot. These results indicate that adventitious rooting in *J. curcas* is under control of photoreceptors and that optimal rooting requires fine-tuning of

the salt concentration, auxin, and cytokinin balance and application of synergistic compounds.

Published in: In Vitro Cellular & Developmental Biology-Plant. 2013. Doi:10.1007/s11627-012-9486-4

Contribution: In vitro tissue culture, statistical analysis, and writing parts of the manuscript

Evaluation of the anti-angiogenic activity of saponins from *Maesa lanceolata* by different assays

Kenn Foubert • Annelies Breynaert • Mart Theunis • Rita Van Den Bossche • Guido R. Y. De Meyer • André Van Daele • **Ahmad Faizal** • Alain Goossens • Danny Geelen • Edward M. Conway • Arnold Vlietinck • Luc Pieters • Sandra Apers

Angiogenesis, in which a vascular network is established from pre-existing vessels, is a complex multistep process. Mechanisms underlying angiogenesis can be investigated using a variety of *in vitro*, *ex vivo* and *in vivo* approaches. Evaluation of several promising plants and plant metabolites, including terpenoids, revealed promising anti-angiogenic activity. Since the maesasaponins displayed anti-angiogenic activity in the chick chorioallantoic membrane (CAM) assay, their activity was further investigated in several test systems. The rat aorta ring assay was compared with the placental vein assay and then selected for the *ex vivo* investigation of the saponins. Besides their effect on the viability of HUVEC, the anti-angiogenic capacity of the compounds was also investigated in an *in vivo* zebrafish assay. The activity of the saponins in the viability assay was more pronounced than in the rat aorta ring assay and similar to the effect observed in the CAM assay. The use of different test systems, however, implies different results in the case of saponins.

Published in: Natural Product Communications. 2012. Volume 7. Issue 9, page 1149-1154

Modulation of triterpene saponin production: In vitro cultures, elicitation, and metabolic engineering

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Saponins are secondary metabolites that are widely distributed in the plant kingdom and are often the active components in medicinal herbs. Hence, saponins have a potential for the pharmaceutical industry as antibacterial, virucidal, anti-inflammatory, and anti-leishmanial drugs. However, their commercial application is often hindered because of practical problems, such as low and variable yields and limited availability of natural resources. In vitro cultures provide an alternative to avoid problems associated with field production; they offer a system in which plants are clonally propagated and yield is not affected by environmental changes. Additionally, treatment of in vitro cultures with elicitors such as methyl jasmonate may increase the production of saponins up to six times. In vitro cultures are amenable to metabolic engineering by targeting specific genes to enhance saponin production or drive production towards one specific class of saponins. Hitherto, this approach is not yet fully explored because only a limited number of saponin biosynthesis genes are identified. In this paper, we review recent studies on in vitro cultures of saponin-producing plants. The effect of elicitation on saponin production and saponin biosynthesis genes is discussed. Finally, recent research efforts on metabolic engineering of saponins will also be presented.

Published in: Applied Biochemistry and Biotechnology. 2011. Volume 164, page 220-237

Contribution: Writing parts of the manuscript.

Supplementary data

Supplementary Table S1 Saponins present in different organs of 3 month old *Maesa* spp. The masses are ordered according to their retention times under the peak of the LC-UV chromatogram.

Species	*m/z* $[M-H]^-$	Leaves		Stem		Root		Saponin
		RT (min)	Peak area	RT (min)	Peak area	RT (min)	Peak area	
M. argentea	1233	24.09	313872	24.11	473132	24.17	83405	Maesasaponin I
	1263	-	-	26.87	22603562	27.13	1223130	
	1437	29.00	8924719	29.50	131601	28.95	3384465	
	1275	29.36	4782610	29.90	7336882	29.22	1448352	Maesasaponin III.2
	1205	28.80	649464	29.21	12202208	28.70	6460664	
	1451	32.28	13802387	33.11	242936	32.15	7120953	
	1219	31.45	1099578	31.98	10471208	31.30	7710098	
	1349	-	-	31.89	2194012	31.19	7265015	
	1289	32.68	8692033	33.40	13648698	32.58	5609891	Maesasaponin IV.3
	1421	32.28	641464	33.20	673572	32.28	10807752	
	1485	33.07	2862760	-	-	-	-	
	1465	34.34	16919636	35.25	337788	34.24	2394776	
	1303	34.82	19445586	35.62	21374222	34.73	2201018	Maesasaponin V.3
	1363	-	-	34.23	3482344	34.12	12966950	
	1231	34.60	131182	34.41	5037436	34.49	15559498	
	1477	36.03	12301511	36.12	237698	36.11	2231394	
	1315	36.58	8878505	36.52	3764439	36.57	1872762	Maesasaponin VI.2
	1479	37.61	8084120	37.29	188634	37.36	1250251	
	1525	38.57	5790102	38.64	40935	38.58	688422	
	1363	39.10	5090127	39.09	920549	39.17	358325	
	1329	39.31	6692197	39.37	3111440	39.42	3374747	
	1377	41.81	3149089	41.67	257489	41.70	159998	
M. balansae	1275	29.80	114854	29.79	751145	29.70	52178	Maesasaponin III.2
	1541	31.35	10125433	31.49	2130112	-	-	
	1469	31.55	4745266	31.58	3175746	-	-	
	1419	31.29	531693	31.32	720434	31.46	206893	

	1351	31.68	62423	31.71	4037962	31.64	8078031	
	1219	31.90	205064	31.93	1441633	31.83	10022568	
	1531	34.14	1872328	34.09	279897	-	-	
	1363	35.43	154875	35.28	9351930	35.14	9520206	
	1231	35.65	302020	35.55	2579204	35.51	16117863	
	1573	35.97	3929760	36.01	388144	-	-	
	1315	37.28	58856	37.67	504558	37.77	601515	Maesasaponin VI.2
	1487	37.94	70,17417	37.95	3576372	38.05	147350	
	1531	38.97	8970067	39.19	1033051	38.54	88211	Maesabalide III
	1509	39.44	16782817	39.49	6376099	39.22	219649	Maesabalide IV
	1495	40.15	3769977	39.88	6690529	40.22	2203919	
	1461	40.42	101785	40.11	2629876	40.46	3181867	
	1489	40.95	10875298	40.93	5510196	41.15	151635	
	1573	41.33	2051768	39.50	153652	-	-	Maesabalide V
	1551	41.89	4599136	41.93	1912960	42.15	29872	Maesabalide VI
M. lanceolata	1233	24.00	212424	24.38	276705	24.05	148457	Maesasaponin I
	1277	29.47	14296800	28.89	6904751	29.55	11117297	
	1305	29.81	565575	-	-	29.99	1129791	Maesasaponin III.1
	1275	29.71	3371026	29.71	9626236	29.43	4734778	Maesasaponin III.2
	1217	32.17	5439702	32.54	2263526	32.19	15898472	
	1247	32.35	5662424	32.73	139583	32.41	1186835	Maesasaponin IV.1
	1317	32.56	754329	-	-	-	-	Maesasaponin IV.2
	1289	32.75	14054609	33.08	16402584	33.04	6478349	Maesasaponin IV.3
	1259	34.98	1904970	35.27	599144	35.02	996844	Maesasaponin V.1
	1231	35.24	1399365	35.50	170162	35.26	19313786	
	1331	35.23	1121961	35.46	59365	-	-	Maesasaponin V.2
	1303	35.40	18420340	35.77	26637845	35.64	18400116	Maesasaponin V.3
	1317	37.96	9139437	38.28	8097337	38.03	8533679	
	1337	37.17	180001	-	-	37.20	597178	Maesasaponin VI.1

	1315	37.64	8118345	37.81	12827500	37.64	8611879	Maesasaponin VI.2
	1345	38.25	3405400	38.47	106771	38.27	789012	Maesasaponin VI.3
	1357	39.99	842675	-	-	40.03	403243	Maesasaponin VII.1
	1313	40.25	633198	40.56	847311	40.27	140633	Maesasaponin VII.2
M. perlarius	1275	29.87	171926	29.86	573922	29.81	34010	Maesasaponin III.2
	1469	31.68	1321297	31.73	3225484	-	-	
	1219	32.03	118592	32.06	799038	32.01	11211745	
	1289	33.52	341026	33.57	1578852	33.55	606549	Maesasaponin IV.3
	1363	35.42	78552	35.37	3935537	35.36	9509265	
	1411	35.41	9033583	35.39	614001	35.32	68207	
	1231	35.68	154471	35.74	2025847	35.61	13198257	
	1303	35.86	1115813	35.89	416730	35.84	479015	Maesasaponin V.3
	1463	35.91	100230	35.57	4517375	35.93	3823053	
	1337	36.88	68472	37.23	404918	37.25	89144	Maesasaponin VI.1
	1315	37.79	405140	37.80	259701	37.82	354456	Maesasaponin VI.2
	1487	38.09	9569257	38.06	2784974	38.18	229456	
	1489	38.06	3417079	38.02	1225710	38.13	83637	
	1531	39.45	7437357	38.92	1652467	39.68	29596	Maesabalide III
	1509	39.86	18933556	39.46	5579182	40.14	582208	Maesabalide IV
	1495	40.20	1626069	40.19	4730328	40.30	2099215	
	1461	40.44	39151	40.62	683477	40.56	2900993	
	1489	41.04	6563270	41.01	2686128	41.20	253892	
	1491	41.06	2306478	41.05	988078	41.21	262898	
	1573	41.23	5601425	41.30	6122303	41.22	57149	Maesabalide V
	1551	41.68	15840130	41.84	15044477	42.10	307547	Maesabalide VI
	1509	43.22	80550	43.26	1794378	43.25	586031	
	1541	47.23	5798776	47.09	355330	47.23	16620